Towards a More Complete Conformational Understanding of the HIV-1 Viral Infectivity Factor

A dissertation presented by

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry and Chemical Biology in the Graduate School of Arts and Sciences of Northeastern University, November, 2011
Every twelve seconds another person contracts the HIV Virus and every sixteen seconds another person dies from AIDS (UNAIDS, 2010). Despite current treatments for the HIV virus, the AIDS pandemic is still a serious threat to human health. Currently, there are large gaps in our biophysical understanding of the processes that underlie HIV infectivity and the viral components responsible for promoting the disease.

One protein that is essential for the productive infectivity of HIV is Vif (Viral Infectivity Factor). Vif, one of the HIV accessory proteins, acts to promote infection by serving as an adaptor molecule which links the potent cellular antiviral APOBEC3G enzyme with the cellular ubiquitin tagging machinery (ubiquitin ligase) that is necessary for protein degradation (Chiu, 2008). Despite the importance of Vif in HIV biology, the structure and conformation of Vif is unknown and the interactions between Vif, APOBEC3G, and ubiquitin ligase are poorly understood. Due to the instability of Vif at concentrations required for traditional biophysical analysis, it is not amenable to X-ray crystallography or NMR spectroscopy. Any biophysical information that could be obtained for Vif either alone or interacting with its cellular partners would be an invaluable and profound contribution to the field of virology.

Hydrogen Exchange monitored by Mass Spectrometry (HX MS) was employed to analyze the conformation and dynamics of recombinant HIV-1 Vif. HX MS analysis of Vif alone in solution revealed that the N-terminal portion of the molecule that contains the APOBEC3 binding region was protected from amide exchange and likely contained the majority of structural elements present in Vif. The C-terminal portion of Vif, which is responsible for association with the ubiquitin ligase machinery, was easily deuterated and was likely unfolded and solvent exposed in solution. Vif conformation was also analyzed in the absence and presence
of the co-factor \( \text{Zn}^{+2} \) which is responsible for mediating the interaction with the ubiquitin ligase component Cullin 5. The region responsible for zinc binding was found to undergo conformational exchange in solution in the absence of zinc which could have resulted from conformational transitions of the Vif monomer or an effect of Vif oligomerization. Upon zinc binding, the conformational exchange was slowed suggesting zinc coordination stabilized Vif.

The conformational consequences of the interaction between Vif and the ubiquitin ligase components Elongin BC were then studied. HX MS revealed that conformational changes occurred in Elongin B and C upon Vif binding. The region in Vif responsible for Elongin BC association, the Vif BC box, was unfolded in the absence of the Elongin BC complex and folded upon incubation. HX MS was also used to probe the affinity of a series of Vif variants for the Elongin BC complex and revealed that removal of the Vif BC box abolished Elongin BC binding.

The information obtained from HX MS analysis of the Vif:Elongin BC interaction was then utilized in the design of hydrocarbon stapled Vif peptide inhibitors targeting the Vif:Elongin BC axis. Peptides were produced using solid phase synthesis, hydrocarbon stapled, and purified successfully. HX MS analysis indicated that hydrocarbon stapling stabilized the peptide into an alpha helical conformation and that stapling did not interfere with Elongin BC binding. Vif peptides were capable of inhibiting the formation of Vif:Elongin BC complex and displayed anti HIV activity.

Lastly, the work with Vif peptides and the Elongin BC complex was employed in the development of a fluorous based flow HX platform using the Vif:Elongin BC complex as a model system. A Vif peptide capable of binding Elongin BC was fluorous tagged and immobilized onto a fluorous surface. The fluorous system was capable of capturing Elongin BC.
from a purified mixture and also from an *E. coli* lysate. Flow HX was conducted on captured Elongin BC and compared to solution HX experiments which yielded similar results illustrating the feasibility of using fluorous chemistry in HX applications.

**(References:**


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I dedicate this work to my mother, Barbara Mary Marcsisin.

August 28th 1943 – April 21st 2011

Nature, nurture, heaven and home
Sum of all and by them driven
To conquer every mountain shown
But have never crossed the river
Braved the forest braved the stone
Braved the icy winds and fire
Braved and beat them on my own
Yet I'm helpless by the river

Angel, angel what have I done?
I've faced the quakes the wind, the fire
I've conquered country, crown, and throne
Why can't I cross this river?

Pay no mind to the battles you've won
It'll take a lot more than rage and muscle
Open your heart and hands my son
Or you'll never make it over the river
It'll take a lot more than words and guns
A whole lot more than riches and muscle
The hands of the many must join as one
And together we'll cross the river

-MJK
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LIST OF ABBREVIATIONS AND SYMBOLS

Å   Angstrom
ACN   Acetonitrile
APOBEC   Apolipoprotein B mRNA editing enzyme catalytic polypeptide-Like
CD_{(followed by number)}   Cluster of differentiation
CD   Circular dichroism
Hck   Hematopoietic Cell Kinase
D   Deuterium
D_{2}O   Deuterium oxide
DIEA   N,N-diisopropylethylamine
DMF   N,N-dimethylformamide
DMSO   Dimethyl sulfoxide
DNA   Deoxyribonucleic acid
E. coli   Escherichia coli
ESI   Electrospray ionization
FBS   Fetal bovine serum
FITC  Fluorescein isothiocyanate
Fmoc  Fluorenylmethyloxycarbonyl
FPLC  Fast protein liquid chromatography
FRET  Fluorescence resonance energy transfer
FTIR  Fourier transform infrared spectroscopy
Fyn   Tyrosine specific kinase Fyn
 g    Gram
GdnHCl Guanidine hydrochloride
GST   Glutathione S-transferase
Hck   Hematopoietic cell kinase
HCTU  \( O-(6\text{-Chlorobenzotriazol-1-yl})\cdot N,N',N''-\text{tetramethyluronium hexafluorophosphate} \)
HeLa  Henrietta Lacks immortal cervical cells
hr    Hour
HIV   Human Immunodeficiency Virus
HPLC  High performance liquid chromatography
IC\(_{50}\) Half maximal inhibitory concentration
IPTG  Isopropylthio-\(\beta\)-D-galactoside
ITC   Isothermal titration calorimetry
\( k \)  Rate constant
kDa   Kilo-Dalton
\( K_d \) Dissociation constant
L     Liter
LC  Liquid chromatography
Lck  lymphocyte-specific kinase
LB   Luria-Broth
Lyn  Tyrosine specific kinase Lyn
mg   Milligram ($10^{-3}$ gram)
min  Minute
mL   Milliliter ($10^{-3}$ L)
mM   Millimolar
mm   Millimeter
MS   Mass spectrometry
MS/MS Tandem mass spectrometry
M    Molar
MW   Molecular weight
Nef  Negative factor
Ni-NTA Nickel-nitrilotriacetic acid
nm   Nanometer
nmol Nanomole
NMR  Nuclear magnetic resonance
OD   Optical density
PAGE Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDB  Protein data bank
<table>
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<tr>
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<tr>
<td>pmol</td>
<td>Picomole ($10^{-12}$ mole)</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Q-Tof</td>
<td>Quadrupole time of flight mass spectrometer</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small angle X-ray scattering</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>sec or s</td>
<td>Second</td>
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<tr>
<td>SFK’s</td>
<td>Src-family kinases</td>
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<td>SH2</td>
<td>Src homology 2 domain</td>
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<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
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<tr>
<td>SOCS box</td>
<td>Suppressor Of Cytokine Signaling box</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
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<tr>
<td>Tof</td>
<td>Time of flight mass spectrometer</td>
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<td>UN</td>
<td>Undeuterated</td>
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<td>µg</td>
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<td>µL</td>
<td>Microliter</td>
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<td>µm</td>
<td>Micrometer</td>
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<tr>
<td>UPLC</td>
<td>Ultra performance liquid chromatography</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral or Virion infectivity factor</td>
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<tr>
<td>VIS</td>
<td>Visible</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
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<tr>
<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
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CHAPTER 1
Strike and Counterstrike: Duel between the HIV Viral Infectivity Factor and APOBEC3 Enzymes

Parts of this chapter have been published in *The Journal of Molecular Biology* which is included in Appendix I:

Special focus issue: Structural and Molecular Biology of HIV

1.A Background and significance.

1.A.1 The Human Immunodeficiency Virus and progression to AIDS

The AIDS pandemic is one of the greatest threats to human health. As of 2010 there were more than 34 million people living with the Human Immunodeficiency Virus (HIV) and an estimated 27 million AIDS related deaths (UNAIDS, 2010) since the discovery of HIV by Sinoussi and colleagues in 1983 (Sinoussi, 1983). The relationship between HIV and the human host is complex and dynamic, as it involves both the human adaptive and innate immune responses and the virus’s strategies at evading detection and clearance. Understanding the fundamental biochemical and biophysical processes that are essential for HIV replication and infection will allow for the design and development of more potent HIV therapies and ultimately vaccinations.

The Human Immunodeficiency Virus belongs to the *Retroviridae* family of viruses and is encoded by a 9 kilo-base genome (Figure 1.1). The HIV-1 genome encodes a total of fifteen proteins; four structural, two envelope, three enzyme, and six accessory (Frankel, 1998). HIV
Figure 1.1 The human immunodeficiency virus-1 genome. The HIV-1 genome shown in cartoon form. The position of the gene encoding the viral infectivity factor (red) is shown in relation to the rest of HIV-1 genes (grey). The 5’ and 3’ prime ends of the genome are capped with long terminal repeats. Adapted from (Frankel and Young, 1998).
differs from primitive retroviruses in that HIV requires the expression of additional proteins besides Gag, Pol, and Env (the main structural, enzymatic, and envelope components, respectively) for efficient and productive viral infection (Frankel, 1998; Seelamgari, 2004). These additional proteins can be classified into regulatory and accessory proteins. The regulatory proteins consist of Tat and Rev which are responsible for viral gene regulation, while the accessory proteins consist of Vif, Vpr, Nef, and Vpu. The accessory proteins play a pivotal role in viral pathogenesis by acting as versatile adaptors that bridge viral and host cellular pathways essential for viral infection and immune evasion (Bour, 2000; Malim 2008). The primary target cells of HIV are those displaying the CD4 cell surface receptor which includes helper T-cells, macrophages, and dendritic cells (Turner, 1999). The hallmark sign of a patient’s progression from just being HIV-1 or 2 positive to AIDS is the depletion of CD4+ cells from normal levels (500-1500 cells/μL) to a level of <200 cells/μL. Depletion of CD4+ cells severely impairs immune system function and patients become susceptible to opportunistic infections and cancers ultimately leading to death (UNAIDS, 2010).

The HIV life cycle (Figure 1.2) begins with virus recognition of the appropriate cell surface receptors on a target cell. The virus fuses with the cellular membrane and the internal components of the virus are released into the cytoplasm. The components include viral mRNA, and the necessary protein machinery for reverse transcription, DNA synthesis, and proviral DNA integration into the host cell chromosome. The steps leading up to proviral DNA integration are termed the early phase of the life cycle. After successful infection the viral life cycle progresses to the late phase which includes viral mRNA transcription, viral protein synthesis, viral particle assembly, and release of infectious viral particles.
Figure 1.2 Lifecycle of the HIV virus. A cartoon depiction of the early and late phases of the HIV-1 life cycle upon infection of a CD4+ target cell. Adapted from (Turner, 1999).
1.A.2 Strike: restriction of HIV by the APOBEC3 enzymes

The investigation of the accessory HIV protein Vif (viral infectivity factor, Figure 1.1, red), in the late 1980’s and early 1990’s, lead to the discovery that Vif is required for viral replication in certain cell types (Fisher, 1987; Strebel, 1987; Gabuzda, 1992). Cells in which Vif was dispensable for replication were termed HIV permissive cells (Figure 1.3A) and included the T cell lines Jurkat and SuptT1. Several non-hematopoietic cell lines were also determined to be permissive such as HeLa, 293T, and COS (Chiu, 2008). Cells in which HIV required the presence of Vif for viral replication included the biologically relevant CD4+ T cells, macrophages, and the T cell leukemia lines H9, HuT78, and MT2 (Chiu, 2008) (Figure 1.3B). This pattern of Vif dependent cell restriction of HIV replication indicated that in HIV non-permissive cell lines there is a mechanism that prevents efficient viral replication. This HIV restriction factor present in non-permissive cells remained to be discovered until the early 2000’s.

In 2003 Sheehy and colleagues discovered that non-permissive HIV target cells express a potent antiviral factor that is suppressed by Vif. This protein, APOBEC3G, which was identified as member of the APOBEC3 family of cytidine deaminases, was found to be expressed exclusively in non-permissive cells thus explaining the Vif dependent cell-specific replication of HIV (Sheehy, 2003) (Figure 1.3C). The APOBEC3 family of cytidine deaminases is a family of zinc dependent RNA editing enzymes which are part of the innate immune response. There are eight known members of the APOBEC3 family (A-H), while only APOBEC3G and F display potent HIV activity (Chiu, 2008). Biochemical exploration of APOBEC3G led to the discovery that upon infection of non-permissive cell lines with ΔVif HIV, APOBEC3G was incorporated into viral particles (see Figure 1.3B) by association with the nucleocapsid region of the HIV gag
Figure 1.3 Cell-specific restriction of HIV infection. (A) When HIV permissive cells (which lack APOBEC3G) are infected with a HIV virus lacking the Vif gene (ΔVif) the resulting viral particles are infectious. (B) HIV non-permissive cells (which express APOBEC3G) infected with a ΔVif HIV virus produce viral particles containing the APOBEC3G enzyme which suppresses further viral infection. (C) The presence of Vif in wild type HIV allows for the degradation of APOBEC3G and the replication of virus in both cell types. Adapted from (Kremer, 2005).
protein and viral RNA (Chiu, 2008). When viral particles containing APOBEC3G infected new target cells, HIV infection was terminated by APOBEC3G’s antiviral activity. APOBEC3G exhibits two known mechanisms of antiviral activity (Figure 1.4). (1) The first mechanism is dependent on the intrinsic deaminase activity of APOBEC3G. APOBEC3G deaminates multiple cytidines in the viral DNA to uridines which results in guanidine to adenine hyper-mutations upon viral DNA second strand synthesis. These mutations are lethal for the virus and cause integration defects, impaired DNA synthesis, and ultimately viral DNA destruction (Chiu, 2008). (2) The second mechanism is deaminase independent and involves inhibition of viral DNA strand transfer during HIV reverse transcription (Li, 2007). Ultimately the combination of APOBEC3G’s antiviral activities renders HIV infection unproductive.

1.A.3 Counterstrike: degradation of APOBEC3 enzymes by HIV Vif

The antiviral activities of the APOBEC3 enzymes have the capability of halting HIV infection; however HIV has evolved the cunning mechanism of utilizing the accessory protein Vif which is capable of counterstriking APOBEC3G activity. Vif is a 22.5 kDa highly basic protein with an iso-electric point of 9.97 (Schröfelbauer, 2004) that interacts with an array of cellular proteins as well as with DNA and RNA [reviewed in (Chiu, 2008; Goila-Gaur, 2008; Barraud, 2008)]. Vif contains no known catalytic activity and instead acts as an adaptor molecule linking cellular pathways required for viral replication. To circumvent the antiviral activities of the APOBEC3G/F cytidine deaminases, Vif binds to APOBEC3G/F and directs them to the Elongin BC-Cullin 5-dependent E3 ubiquitin ligase complex for poly-ubiquitylation. Ubiquitin tagging of APOBEC3G leads to degradation by the 26S proteasome (Yu, 2003; Mehle, Strack, 2004) (Figure 1.5). Vif has also been shown to counteract APOBEC3G by directly inhibiting
Figure 1.4 Restriction of HIV infection by the APOBEC3 enzymes. APOBEC3G displays potent anti-HIV activity by two separate mechanisms: deaminase dependent and deaminase independent mechanisms. Rendition of the HIV-1 Virus created by David S. Goodsell (http://mgl.scripps.edu/people/goodsell/illustration/public).
Figure 1.5 Degradation of APOBEC3G by the HIV viral infectivity factor. HIV-1 Vif (red) counteracts the antiviral activities of APOBEC3G (blue) by directing it for Proteasomal degradation. Vif acts as an adaptor molecule linking the APOBEC3 enzymes with the E3 ubiquitin ligase machinery (green). Upon ubiquitylation APOBEC3G is degraded by the proteasome. Adapted from (Goila-Gaur, 2008).
APOBEC3G mRNA translation and promoting the formation of inactive high molecular weight complexes (Mercenne, 2010; Stopak, 2003; Golia-Gaur, 2008).

Despite the importance of Vif in the suppression of the APOBEC3 enzymes, there is a lack of structural knowledge on full-length Vif alone or in complex with cellular factors such as APOBEC3G, Elongin BC, or Cullin 5. To illustrate the domain layout of Vif and the corresponding biological functions of each region, consider Figure 1.6A which shows a cartoon depiction of HIV-1 Vif. The N-terminal portion of the protein contains the APOBEC3G/F binding sites (blue). The central portion of the molecule is responsible for binding zinc and association with the E3 ligase component Cullin 5 (black). The C-terminal portion (red, orange, and green) of Vif is responsible for binding with Elongin BC, Gag, NCp7, cellular membrane, the tyrosine kinase Hck, and multimerization [see (Mehle, Goncalves, 2004; Stanley, 2008; Bernacchi, 2010; Zhang, 2000; Hassiane, 2001; Wissing, 2010)]. Structural prediction algorithms such as PONDR (Li, 1999; Romero, 1997; Romero, 2001) predict regions of disorder as well as structured segments. The only three dimensional structural information for Vif is from an X-ray structure containing Vif residues 140-156 bound to the E3 ubiquitin ligase component Elongin BC complex (described in depth in Chapter 4) as shown in Figure 1.6B (Stanley, 2008). This structure led to the modeling of residues 140-175 with the E3 ligase components Elongin BC and Cullin 5 (Stanley, 2008). Understanding the structural mechanism(s) by which Vif assembles the E3 ligase complex would provide insight into how HIV manipulates human host cell machinery for protein degradation as well as providing information from which Vif inhibitors could be designed.

Besides the crystallographic data for Vif and structural predictions, there have been several attempts to produce structural homology models and numerous studies on the different
Figure 1.6 Selected HIV-1 Vif conformational knowledge. (A) Shown in cartoon form are the biologically relevant regions of Vif, adapted from (Marcislin, 2011). Amino acids are highlighted to show the relative positions. Below the cartoon is the PONDR structural prediction for Vif. A PONDR score of < 0.5 indicates regions that are likely structured while a score of > 0.5 indicates regions that are likely unstructured. (B). Crystal structure (PDB:3CDG) of Vif residues 140-156 (red) bound to the Elongin BC complex (yellow and green) and C. Model of the Vif SOCS box (red) with Elongin BC (yellow and green) and Cullin 5 (grey) (Stanley, 2008).
segments of Vif including the zinc finger, APOBEC3 binding region, and C-terminal portion. Several studies by Giri et al utilized synthetic peptides approximating the zinc binding region of Vif and probed the conformational consequences of zinc binding with circular dichroism, dynamic light scattering, XAS, and fluorescence microscopy (Giri, 2009; Giri, Scott, 2009). These studies indicated that the zinc binding region in Vif was unstructured in the absence of zinc. The work of Reingewertz et al probed the C-terminal region of Vif using similar strategies to those above for the zinc binding region and determined this region to lack secondary structural elements like alpha helices and beta sheets in solution in the unbound state (Reingewertz, 2009).

The studies of Wolfe et al and Bergeron et al used a small peptide approximating the Elongin BC binding domain (~ 15-30 residues) of Vif to probe the assembly of the Vif:Elongin BC complex (Wolfe, 2010; Bergeron, 2010). Using a combination of NMR and ITC they were able to determine that the Elongin BC binding domain was unstructured in the absence of Elongin BC, and only folds upon binding.

The only published attempt at the biophysical analysis of full-length Vif was conducted by Auclair et al (Auclair, 2007). They utilized chemical cross-linking and mass spectrometry to probe the conformation of recombinant Vif. The results obtained from the cross-linking experiments provided some insight into how Vif was folded in solution. The cross-linking results and homology models proposed by Balaji et al and Lv et al (Balaji, 2006; Lv, 2007) will be discussed in more detail in relation to HX MS results for Vif in Chapter 3. The results provided by the HX MS conformational analysis of full-length Vif presented in this dissertation along with the work of the groups described above provide the HIV community with new insights into how this essential HIV accessory protein is folded in solution.
1.B Approach to analysis and dissertation overview

1.B.1 Research Aims and HX MS analysis

To date there is no three dimensional structure of full-length HIV Vif. Vif likely contains multiple disordered regions and is prone to aggregation making structural characterization with more commonly used techniques such as X-ray crystallography and NMR difficult. An alternative biophysical technique that can be used to gain conformational information for proteins that are difficult to study otherwise is amide hydrogen exchange monitored by mass spectrometry (HX MS). Using HX MS as the primary tool for conformational investigation, the primary research Aims of this work were (Figure 1.7):

(1) Probe the solution conformation of HIV-1 Vif with HX MS.
(2) Use HX MS to probe conformation of Vif and E3 ligase components upon binding.
(3) Test inhibitors towards the Vif:Elongin BC complex and the development of Elongin BC as a model system for HX fluorous capture.

Advantages of HX MS that make it well suited for Vif conformational analysis is that (1) very little material is required (as little as 500-1000 pmol for an entire experiment) and (2) the concentration of the protein can be low (as low as 0.1 μM) as compared to the concentrations required for NMR and X-Ray analysis (≥ 0.5 mM) (Engen, 2009). This technique allows for the analysis of proteins such as Vif that are prone to aggregate, difficult to purify, and/or otherwise hard to obtain in suitable quantities for biophysical characterization by other methods. One disadvantage of HX MS is that it cannot be used to solve the structure of a protein; however it can be used to provide some conformational information, detect conformational changes, and differentiate protein subpopulations in solution (Engen, 2009). A detailed explanation of the theory and workflow for HX MS will be provided in Chapter two.
Figure 1.7 Aims of this research. The first aim was to obtain conformational information on Vif and determine if Vif was unstructured or contained structured regions and where structure was. The second aim was to explore the binding between Vif and cellular factors Elongin BC and Src family kinases. The third aim was to design and synthesize HIV-1 Vif inhibitors and use the Vif:Elongin BC complex as a model system to test fluorous “capture” chemistry.
1.B.2 Dissertation overview

The overall goals of Aims 1 and 2 of this research were to ascertain how Vif folds, moves, and breathes in solution and how this behavior changes upon interaction with different cellular components. In order to complete aims 1 and 2, Vif needed to be produced recombinantly in *E. coli*. Chapter 2 describes the unifying material and methods utilized throughout this dissertation including Vif expression and purification and conformational analysis by HX MS. The two expression and purification protocols used for obtaining recombinant Vif from *E. coli* are outlined which included the expression and purification of Vif as: (1) a GST-fusion protein and (2) the expression and purification of Vif using a denaturing purification followed by slow protein refolding using dialysis. The denaturing purification was successful and produced protein for conformational analysis using circular dichroism (CD) and HX MS (as described in a Chapter 3). Since HX MS was used extensively throughout this work, a detailed explanation of the background and theory for the method is presented in the second half of Chapter 2. The mechanisms governing amide exchange are described along with the different factors that dictate hydrogen exchange in proteins. The detection of hydrogen exchange with mass spectrometry, data analysis methods, and sources of error are also discussed in Chapter 2.

In order to assay the functionality of recombinant Vif, several binding assays between Vif and the previously identified binding partner, the SH3 domain of the Hematopoietic Cell Kinase (Hck) (Hassaine, 2007) are presented in Chapter 3. Results indicate that Vif purified under denaturing conditions interacted specifically with the Hck SH3 domain in solution, as expected. The implications of the Vif:Hck interaction on Hck kinase activity were assayed using an in vitro
kinase assay which suggests that Vif was a potent activator of kinase activity in vitro. The results presented in Chapter 3 show that Vif retained a biochemically competent conformation even when re-natured. The second part of chapter 3 includes the biophysical analysis of HIV-1 Vif using HX MS and circular dichroism. CD analysis of Vif showed that the majority of Vif secondary structure was beta sheet while HX MS revealed that Vif displayed protection from exchange in the N-terminal region which is responsible for association with the APOBEC3 enzymes. The C-terminal region, which is responsible for E3 ligase binding, was easily deuterated indicating that this region was solvent exposed and likely did not contain stable tertiary structure in solution.

In Chapter 4 the biophysical consequences of Vif associating with the E3 ligase components (Elongin BC) are presented. The Elongin BC complex was produced recombinantly and assayed for binding using pull-down analysis and HX titration assays against a series of Vif constructs. Unlike previous Elongin BC binding proteins, such as cellular SOCS (Suppressor Of Cytokine Signaling) 1-7, Vif interacted with Elongin BC in vitro without the aid of other cellular components or folding chaperones. The apparent Vif:Elongin BC affinity was diminished 2-fold with mutations in the Vif SOCS motif and completely abolished when the SOCS box was removed. Intact HX MS analyses of all three components together as compared to alone revealed no significant changes in Vif conformation, large changes in Elongin C conformation, and slight changes in Elongin B conformation upon complex formation. A Vif SOCS peptide (Vif^{135-158}) was also probed with HX MS which was unfolded in the absence of the Elongin BC complex and folded upon binding. Peptic peptide HX MS analyses of Elongin allowed for the localization of conformational changes upon binding HIV-1 Vif. These changes localized to the Vif:Elongin C binding interface as suggested by the Vif:Elongin BC crystal structure (PDB:3DCG) (Stanley,
2008). The results obtained and described in this Chapter provide new insight into the assembly of the Vif:Elongin BC complex.

In Chapter 5 the design and synthesis of inhibitors towards the Vif:Elongin BC complex is described. Results obtained from screening a peptide library derived from Vif sequences revealed the minimal length peptide required to bind the Elongin BC complex in vitro as well as demonstrating that a short Vif peptide could displace a reporter molecule bound to the Elongin BC complex. Using the information from the Vif peptide screen, a hydrocarbon stapled Vif peptide was designed and synthesized. The stapled peptide was assayed with HX MS and CD which revealed that hydrocarbon stapling stabilized the helicity and peptide conformation. Hydrocarbon stapling did not interfere with binding as incubation of the stapled Vif peptide with the Elongin BC complex resulted in deuteration changes in the Elongins consistent with binding as observed for full-length wt Vif. To test the ability of the stapled peptide to enter cells, a fluorescently labeled peptide was produced and assayed using confocal fluorescence microscopy and fluorescence activated cell sorting. Results from both methods suggested that the stapled peptide rapidly and efficiently entered HeLa cells. This work provides a basis for further rational design of Vif:Elongin BC peptide inhibitors.

The work described in Chapter 6 uses the Vif_{138-161} peptide and the Elongin BC complex as a model system to test a fluorous capture system for HX applications. Fluorous chemistry takes advantage of the fluorophilic nature of poly-fluorinated compounds. For this work a fluorous tagged Vif_{138-161} peptide was produced that could be immobilized onto fluorous silica packed into column format. The Vif_{138-161} probe was then used to “capture” Elongin BC produced recombinantly as well as Elongin BC from a crude E. coli lysate. HX MS analysis was conducted on Elongin BC in solution as well as on the fluorous column which yielded similar
results. A flow system was designed, built, and tested to allow for the automation of an HX fluorous capture system. This work demonstrates that the fluorous tag/capture system allows for the selective capture of a fluorous probe and its target protein from a complex mixture. The conformation of the protein complex can then be analyzed with HX MS.

Chapter 7 highlights the future direction of the work presented in Chapters 1-6. These future directions include the completion of the comprehensive investigation of Vif with key cellular components such as APOBEC3G.

1.C Summary

The work presented in this dissertation on the HIV-1 viral infectivity factor provides new insight into this HIV-1 accessory protein. The fundamental conformational knowledge obtained for Vif provides insight into how the protein is folded in solution as well as how Vif interacts with the cellular components Elongin BC. This information also allows for the practical design screening of Vif:Elongin BC inhibitors as well as uses in HX fluorous capture applications. The work described provides the basis for future work to ultimately understand the mechanisms governing Vif conformation and Vif complex assembly.

1.D References


CHAPTER 2
Unifying Materials and Methods for the
Conformational Analysis of Vif

Parts of this chapter have been published and are included in Appendix I and II:


2.A Introduction

Throughout this dissertation there were several unifying methods which included Vif protein production and conformational analysis by HX MS. The source of HIV-1 Vif (protein production) and the main method chosen for conformational analysis (HX MS) will be discussed in this Chapter. The first part of this Chapter describes the work to obtain soluble recombinant HIV-1 Vif in quantities suitable for HX MS analysis. The second part of this Chapter describes the background, theory, and workflow of HX MS which is necessary to understand the results presented in future Chapters.

2.B Vif protein expression and purification

To complete Aims 1 and 2 described in Chapter 1, HIV Vif needed to be expressed and purified. Sections 2.A.1 and 2 of Chapter 2 describe the two different strategies utilized to produce Vif recombinantly. The first strategy involved producing Vif as a GST-fusion protein and purifying under non-denaturing conditions. Expression and purification of both HIV-1 and 2 Vif proteins were attempted in order to compare their conformations. The reason why the HIV-1
and 2 Vif proteins were chosen for comparison was that both perform the same function: targeting the APOBEC3 enzymes for proteasomal degradation, however, differ significantly in their primary structure. The HIV-2 Vif gene has only 25% identity to the HIV-1 Vif gene (Ribeiro, 1998). The conformation of both Vif proteins was to be compared using HX MS after production to ascertain how proteins with different primary structures performed the same function.

The first strategy for making Vif produced large amounts of protein aggregates and required non-mass spectrometry compatible detergents to obtain small amounts of usable Vif. Due to the low yields and the presence of detergents, the first strategy was abandoned for the second, which involved producing Vif from a codon optimized expression vector and purifying under denaturing conditions. Purified denatured Vif was then renatured successfully by serial dialysis against buffers containing decreasing concentrations of denaturant. This method produced Vif that was used throughout the work presented in future chapters.

2.B.1 Production of HIV-1 and 2 Vif as GST-tagged proteins

To produce HIV-1 and 2 Vif as GST-fusion proteins, the HIV-1 pGEX-2T and HIV-2 pGEX-6P-1 expression vectors were obtained from Professor Dana Gabuzda at the Dana Farber Cancer Institute. The pGEX vectors were transformed into Rosetta 2 (DE3) pLysS competent cells and plated on Luria broth (LB) agar plates containing chloramphenicol and ampicillin (Figure 2.1A). Resulting colonies were selected and used to inoculate 50 mL LB starter cultures for protein expression. After an overnight incubation, starter cultures were used to inoculate 500 mL of LB in a 2 L flask. The resulting cultures were grown at 37 °C until optical density (OD$_{600}$ nm) values of $\geq 0.8$ were reached. Two separate expression conditions were tested after the
Figure 2.1 Expression of HIV Vif using the GST-fusion system. (A) The Vif gene in the pGEX-2T expression vector was transformed into Rosetta 2 (DE3) pLysS cells. (B) Protein expression was monitored using SDS PAGE. Aliquots of cells expressing GST-Vif were taken at different times throughout the induction and compared to cells prior to incubation with IPTG (TO). The band corresponding to HIV-1 GST-Vif is indicated with the red asterisk.
addition of 100 µM Isopropyl β-D-thiogalactoside (IPTG) to the E. coli cultures which initiates protein expression. Cultures were then either grown at 37 °C for 5 hours or 18 °C for 20 hours. Aliquots of the induction were taken throughout the duration of experiment and analyzed with SDS-PAGE as shown in Figure 2.1B. The gel in Figure 2.1B shows the expression of GST-HIV-1 Vif at 18 °C for 20 hours. The different expression times indicated were compared to the un-induced sample (TO) which provided a snapshot of protein levels prior to initiation of protein expression. A band approximately 50 kDa in molecular weight increased with time and was believed to be GST-Vif as indicated by the red asterisk in Figure 2.1B. Successful expression of both HIV-1 and 2 (data not shown for HIV-2 Vif) at 37 °C for 5 hours and 18 °C for 20 hours was observed. After expression, cells were centrifuged, supernatant discarded, and cell pellets frozen at -80 °C until purification.

Affinity purification of GST-Vif relied on the affinity of the GST-tag for glutathione as depicted in Figure 2.2. Cells containing GST-Vif were lysed using sonication and one of two different lysis buffers. The first buffer contained 1 M NaCl, 10% glycerol, 50 mM Tris, pH 7.0, while the second contained 2% of the detergent η-dodecyl β-D-maltoside to aid with protein solubilization. After lysis, the lysate was centrifuged at 20,000 x g for 40 minutes at 4 °C to remove any insoluble components and allow for further purification steps. The supernatant was then incubated with reduced glutathione (GSH) agarose for 1 hour at 4 °C. Contaminant proteins, such as endogenous E. coli proteins, were washed away using 30 mL of Lysis buffer. GST-Vif was eluted from the GSH agarose by incubation with a solution of 250 mM reduced GSH. GST-Vif elution was monitored by SDS-PAGE and purified protein was either stored at -80 °C or subjected to protease removal of the GST affinity tag. The purpose of removing the GST tag was
**Figure 2.2 GST-Vif purification protocol.** Two different methods were used to lyse cells containing recombinant GST-Vif. Both methods used sonication for cell disruption, despite utilizing different lysis buffers. The soluble protein fraction was isolated by centrifugation and applied to GSH agarose. Contaminant proteins were then washed away from the agarose slurry leaving purified GST-Vif which was then eluted with a solution containing reduced GSH. The eluted GST-Vif was either analyzed directly or subjected to protease removal of the GST affinity tag.
Figure 2.3 Purification gels of GST Vif. (A) Purification gel of HIV-1 Vif expressed at 37 °C and purified without detergent. The Vif induction sample was used as a reference as it contains the GST-Vif band (red asterisk). Vif produced and purified in this manner remains insoluble (indicated by the band disappearance after the insoluble pellet lane). (B) Gel of purified HIV-1 GST-Vif and (C) HIV-2 Vif expressed at 18 °C and purified with detergent. (D) Factor Xa cleavage of GST from HIV-2 Vif. The GST-Vif input is shown before and after the cleavage reaction. After cleavage, Vif becomes insoluble as indicated by the lack of a band corresponding to cleaved Vif.
to ensure that any conformational measurements made on Vif produced in this manner were not altered by the presence of the large 26 kDa GST tag.

Production of GST-Vif in *E. coli* for biophysical analysis proved challenging and had limited success. Figure 2.3 shows selected results to illustrate the purification process for HIV-1 and 2 GST-Vif (not all data shown for purifications). The attempts to purify GST-Vif expressed at 37 °C for 5 hours were unsuccessful as Vif formed detergent insoluble inclusion bodies. Samples were taken and analyzed with SDS-PAGE from each step of the purification process. The red asterisk in Figure 2.3A indicates the band corresponding to HIV-1 GST-Vif and it was used as a positive control. The GST-Vif band is only apparent in the insoluble protein fraction (green asterisk, Figure 2.3A) after lysis indicating that Vif remained insoluble during the purification process.

The only successful purification of GST-Vif was obtained using protein expressed at 18 °C for 20 hours and purified using detergent as shown for HIV-1 and 2 GST-Vif in Figure 2.3B and C. Both HIV-1 and 2 GST-Vif co-purified with other proteins which were likely the *E. coli* chaperone proteins DnaK, HscA, and/or HscC. The purified HIV-1 and 2 GST-Vif shown in Figure 2.3B and C were subjected to protease removal of GST. Figure 2.3D shows the results for Factor Xa cleavage of GST from HIV-2 Vif. The first lane in Figure 2.3D contained intact HIV-2 GST-Vif before the Factor Xa cleavage and the second lane is after the protease reaction. The protease reaction was unsuccessful at removing Vif from GST as removal of GST resulted in the visual appearance of Vif aggregates. The lack of a band for Vif in Figure 2.3D indicated that Vif produced as a GST-fusion protein was unstable upon GST removal.

To determine if the GST-Vif that was expressed at 18 °C for 20 hours and was purified with detergent could be used for biophysical analysis, 200 picomoles of HIV-2 GST-Vif were
analyzed by LC ESI-MS. The mass spectrum of GST-Vif is shown in Figure 2.4. The charge envelope of GST-Vif can be seen in the resultant spectrum; however, the majority of MS signal is dominated by signal from \( \eta \) dodecyl \( \beta \)-D-maltoside. The difficulties producing soluble stable protein and the overwhelming \( \eta \) dodecyl \( \beta \)-D-maltoside MS signal made HX MS analysis of GST-Vif impractical. Therefore, the expression and purification of Vif as a GST-fusion protein was abandoned.

### 2.B.2 Production of HIV-1 and 2 Vif without affinity tag

Another strategy that was used previously to isolate Vif for biochemical analysis is the protocol described in Yang et al (Yang, 1996). In this protocol, Vif is over-expressed and forms insoluble inclusion bodies containing highly pure protein. Vif was subsequently purified with denaturation and renatured by serial dialysis.

To produce HIV-1 Vif using the Yang et al method, the HIV-1 codon optimized Vif gene in the expression vector pET-28b was obtained from Professor Dana Gabuzda at the Dana Farber Cancer Institute. Vif expressed from the pET-28b expression vector did not contain any affinity purification or solubilization tags. The pET-28b vector was transformed into Rosetta 2 (DE3) pLysS competent cells and plated on agar plates containing kanamycin (Figure 2.5A). Resulting colonies were selected and used to inoculate 50 mL Luria broth starter cultures for protein expression. After overnight incubation at 37 \(^\circ\)C, starter cultures were used to inoculate 500 mL of Luria broth in a 2 L flask. The resulting cultures were grown at 37 \(^\circ\)C until OD\(_{600\text{nm}}\) values of \( \geq 0.8 \) were reached. IPTG was added to the cultures to 100 \( \mu \)M, which were then allowed to grow at 37 \(^\circ\)C for 5 hours. Aliquots of the reactions were taken throughout the duration of expression and analyzed with SDS-PAGE as shown in Figure 2.5B. The gel in Figure 2.5B
Figure 2.4 ESI mass spectrum of HIV-2 GST Vif. 200 picomoles of GST-Vif were analyzed with ESI-MS. The majority of MS signal was dominated by the detergent molecule dodecyl β-D-maltoside.
Figure 2.5 Expression of Codon optimized Vif. (A) The codon optimized Vif gene was subcloned into the pET-28b expression vector, under the control of the T7 polymerase promoter (a kind gift from Dana Gabuzda, DFCI) and transformed into *E. coli*. Protein expression was initiated by addition of IPTG and allowed to proceed for 5 hours at 37 °C. (B) Protein expression was monitored using SDS PAGE. Aliquots of cells expressing Vif were taken at different times throughout the experiment and compared to cells prior to IPTG incubation (TO). The band corresponding to HIV-1 Vif is indicated with the red asterisk.
shows a band between 20 and 25 kDa believed to be Vif (22 kDa, red asterisk). These data indicate the successful expression of Vif. Post expression, cells were centrifuged, and cell pellets were frozen at -80 °C until purification.

The codon optimized HIV-1 Vif that was produced as described above, was purified using the scheme shown in Figure 2.6. Cells containing Vif were lysed by sonication and lysis buffer containing 6 M guanidine hydrochloride (150 mM NaCl, 10% glycerol, 20 mM HEPES, 1 mM DTT, pH 7.0). After lysis, the lysate was centrifuged at 20,000 g for 40 minutes at 4 °C to remove highly insoluble components and genetic material. The supernatant was incubated with Ni$^{2+}$-NTA agarose for 1 hour at 25 °C. Contaminant proteins were then washed away using 30 mL of lysis buffer. Vif was eluted from the Ni$^{2+}$-NTA resin using a step gradient of lysis buffer with decreasing pH values (pH 6.5, pH 6.0, pH 5.5, pH 5.0). Fractions containing denatured Vif were pooled and diluted so the protein concentration was below 35 µM, as determined by Bradford assay (Bradford, 1976). Diluted Vif was renatured by serial dialysis against Vif buffer (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol pH 7.0) for 1.5 hours each with Vif buffer containing 3.0, 1.5, 0.75, 0.21, and 0 M guanidine HCl, respectively. A second 0 M guanidine HCl dialysis was conducted overnight to ensure the complete removal of denaturant. The final dialyzed material was stored at -80 °C until biophysical analysis.

The purification process was monitored with SDS-PAGE and results shown in Figure 2.7. Figure 2.7A shows the lysis and centrifugation results. The 4 hour induction sample (Figure 2.5B) was used as a control and indicates the location of the Vif band (red asterisk). The gel in panel A shows that Vif was present in both the insoluble protein fraction as well as the soluble fraction. After incubation of the Vif lysate with agarose, the Vif bound to the Ni$^{2+}$-NTA resin was incubated with the elution chemical imidazole (Figure 2.7B). Ni$^{2+}$-NTA affinity
Figure 2.6 Codon optimized HIV-1 Vif purification protocol. Cells containing insoluble recombinant Vif were lysed using sonication in lysis buffer containing 8M guanidine HCl. The solubilized protein fraction was isolated by centrifugation and applied to pre-swollen Ni-NTA agarose. Contaminant proteins were then washed away from the agarose slurry leaving purified Vif which was then eluted using a step pH gradient. The eluted denatured Vif was then renatured by dialysis using buffers with decreasing concentrations of guanidine hydrochloride.
Figure 2.7 Purification gels of codon optimized HIV-1 Vif. (A) Purification gel of HIV-1 Vif. The Vif induction sample was used as a reference as it contains the Vif band (red asterisk). (B) Vif remained on the Ni-NTA agarose even after incubation with the eluting chemical imidazole. (C) Vif eluted from the Ni-NTA after using a decreasing pH step gradient.
chromatography is commonly used to purify poly-histidine (6X His) tagged proteins because poly-histidine coordinates the Ni\textsuperscript{2+} ion. Imidazole resembles the side chain of histidine and typically elutes proteins from Ni\textsuperscript{2+}-NTA agarose. The imidazole procedure was unsuccessful at liberating Vif from the Ni\textsuperscript{2+}-NTA as indicated by the lack of Vif bands in the imidazole elution lanes in Figure 2.7B. The alternative strategy of using a decreasing pH gradient was used to elute Vif. Decreasing the pH below the pKa value (6.0) of histidine (Horton, 2002), results in protonation of the amino acid side chain. Protonation of histidine disrupts the interaction between the histidine and the Ni\textsuperscript{2+} ion and was successful at eluting Vif off of the Ni\textsuperscript{2+}-NTA agarose as shown in Figure 2.7C. The ability to purify Vif with Ni\textsuperscript{2+}-NTA agarose without the aid of a poly-histidine tag can likely be attributed to coordination of the Vif zinc finger (see Chapter 1, Figure 1.6A, and Chapter 2, Figure 2.6) with the Ni\textsuperscript{2+} ion. Vif was eluted and renatured by dialysis and the mass of Vif verified with ESI-MS as shown in Figure 2.8. The charge state distribution for Vif is clearly seen as well as the deconvoluted mass (Figure 2.8 inset, 22512.1 Da) which closely matched the theoretical mass (22512.9 Da). The working concentrations for Vif produced in this manner needed to be below 35 \(\mu\text{M}\) to avoid aggregation.

**2.C. Overview of hydrogen exchange: what is it and why use it for Vif?**

The aggregation tendencies of Vif have hampered conformational analysis using traditional biophysical methods such as NMR and X-ray crystallography and therefore these methodologies could not be used to probe the conformation of Vif produced in the work presented. The difficulty of producing HIV Vif for biophysical analysis with X-ray crystallography or NMR prompted the use of amide hydrogen exchange monitored by mass spectrometry. HX MS is well suited for proteins that are prone to aggregation and difficult to purify such as Vif. But, what is HX MS? To address what HX MS is, the theory and background
Figure 2.8 ESI mass spectrum of codon optimized HIV-1 Vif. Vif (200 picomoles, material from Figure 2.7C) was analyzed with ESI-MS. Shown are several charge states of Vif and the deconvoluted measured mass (inset). The presence of an unknown species (27 kDa) is indicated by the grey dots and TFA adducts are shown in the deconvoluted spectrum.
regarding amide hydrogen exchange in proteins and detection with mass spectrometry will be presented in the next pages.

The concept of amide hydrogen exchange as a probe for protein dynamics and structure was first employed in the 1950’s by Kaj Ulrik Linderstrøm Lang at the Carlsberg laboratories in Copenhagen, Denmark. The Carlsberg Laboratories were founded for the advancement of beer brewing and it was there at the Carlsberg Laboratories that Linderstrøm Lang initially realized that protein backbone amide hydrogens participate in continual exchange with protons from the solvent [see (Englander, 1997; Engen, 2009) for historical perspectives]. Linderstrøm Lang made the first measurements of protein deuteration using density gradient tubes (Hvidt, 1954). It was not until 1991 that mass spectrometry was used to monitor hydrogen exchange of proteins (Katta, 1991). The work of Viswanatham Katta and Brian Chait was the first to demonstrate hydrogen exchange mass spectrometry could be used to monitor global protein conformation.

Today the “conventional” hydrogen exchange reaction workflow is shown in Figure 2.9. The workflow consists of labeling a protein of interest with D₂O for various labeling time points at physiological pH and room temperature. The hydrogen exchange reaction is then slowed to a minimum (“quenched”) by dropping the pH and temperature of the reaction matrix. After the solution conformational information has been captured, the protein is either directly analyzed with liquid chromatography and mass spectrometry (LC-MS) or digested with acid proteases prior to LC-MS analysis to allow for the localization of deuterium incorporation along the primary structure of the studied protein. Deuterium incorporation curves are one common output of hydrogen exchange mass spectrometry experiments. Protein dynamics and solvent accessibility, which are dictated by protein structure and/or lack there of, are the two major factors that dictate hydrogen exchange in folded proteins. Deuterium incorporation curves often
Section 2.C

Equilibrate 25°C
desired pH

D²O

D²O

D²O

Deuterium labeled
backbone amide hydrogens

Section 2.D

Quench reaction at
various times,
0°C pH 2.5

2 hr

10 min

1 min

10 sec

pepsin digestion
0°C pH 2.5

or

Intact protein

Figure 2.9 HX MS experiment workflow. Section 2.B of chapter 2 describes the background and theory of the deuterium labeling process for proteins. Section 2.C of chapter 2 outlines the sample processing and LC-MS analysis of deuterium labeled proteins. Section 2.D describes HX MS data analysis and interpretation in relation to protein structure, function, and dynamics. Adapted from (Marcisin, 2010).
allow for the interpretation of protein dynamics, and solvent accessibility which provides insight into how a protein behaves in solution. The subsequent sections of this chapter will highlight in detail the different aspects of the hydrogen exchange in proteins and detection with mass spectrometry.

2.C.1 Hydrogens in proteins

Hydrogen exchange exploits a fundamental chemical reaction unique to certain hydrogens found in proteins. Figure 2.10 illustrates the different hydrogens found in a polypeptide chain. Hydrogen atoms (gray) covalently bonded to carbon atoms essentially do not exchange. The hydrogen atoms (black) bonded to nitrogen, oxygen, and sulfur of the amino acid side chains have some of the fastest exchange rates; becoming deuterated during the exchange reaction and then exchanging back to hydrogen upon quenching and subsequent LC-MS analysis. The hydrogen atoms (blue) bonded to the amide nitrogen of the polypeptide backbone have moderate exchange rates and are retained during analysis under quenching conditions. These amide hydrogens are the atoms that can be used as a probe for protein conformation. The reasons for the difference in proton exchange rates in proteins will be described in Section 2.C.2. Since every amino acid except proline (orange nitrogen) has a backbone amide hydrogen, there is essentially a sensor at every amino acid along the length of a polypeptide chain.

2.C.2 Mechanism of proton transfer

In order to understand the different exchange rates of protons found in proteins as described above, the hydrogen exchange reaction needs to be broken down into its simplest form. The hydrogen exchange reaction can be viewed as a reaction between a hydrogen donor and
Figure 2.10 Hydrogens in proteins. Backbone amide hydrogens (blue) exchange with deuterons from the solvent (red) which are essentially retained during the liquid chromatography step. Hydrogens bonded to oxygen and nitrogen on amino acid side chains (black) also exchange with deuterium, however, revert back rapidly to hydrogen during the liquid chromatography step. Hydrogens covalently bonded to carbon (gray) essentially do not exchange.
hydrogen acceptor. This concept is illustrated in Figure 2.11A, and for dissolved macromolecules in solution at physiological pH, it is driven primarily by the OH\(^-\) ion (Brier, 2008). The rate constant for the proton exchange reaction can be calculated using Equation 2.1 (Brier, 2008).

\[
k = k_1 \left( \frac{10^{(pK_{a\text{acceptor}} - pK_{a\text{donor}})}}{10^{(pK_{a\text{acceptor}} - pK_{a\text{donor}})} + 1} \right)
\]  

(2.1)

The rate constant of exchange \((k)\) is calculated using the diffusion-limited collision rate constant \((k_1)\) \((10^{10} \text{M}^{-1} \text{S}^{-1})\) (Englander, 1972) and the difference between pKa values (value for deprotonation) of the proton donor (A in Figure 2.11A) and acceptor (B in Figure 2.11A). If the proton transfer is from a strong acid to a weaker acid \((pK_{a\text{donor}} > pK_{a\text{acceptor}})\), the rate constant of exchange is close to the diffusion-limited collision rate constant, meaning every interaction between the proton donor and acceptor leads to successful proton transfer. If \(pK_{a\text{donor}} \ll pK_{a\text{acceptor}}\), then the rate constant of exchange will be significantly lower than the diffusion-limited collision rate constant. The main determining factor of the rate of exchange is \(\Delta pK_a\). To illustrate the importance of \(\Delta pK_a\) on proton transfer consider the transfer of side chain protons to OH\(^-\) anions: the pKa value of deprotonation for the OH\(^-\) ion is 15.7, while the pKa value for hydrogens on amino acid side chains is less than 13 (Englander, 1972; Englander, 2006). Using Equation 2.1, then the result is a rate constant value of \(9.9 \times 10^9 \text{M}^{-1} \text{sec}^{-1}\), which is close to the diffusion-limited collision rate constant. The rate constant is much different when calculated for the transfer of amide protons to the OH\(^-\) ion. Amide hydrogens have pKa values around 18 (Molday and Kallen, 1972), which results in a rate constant value of approximately \(4.9 \times 10^7 \text{M}^{-1} \text{sec}^{-1}\). This is two orders of magnitude lower than proton transfer between side chain hydrogen atoms and the OH\(^-\) ion. Hydrogens covalently bonded to carbon do not exchange with solvent
Figure 2.11 Base catalyzed mechanism of amide hydrogen exchange. (A) Hydrogen exchange is the transfer of a proton (blue H) or deuteron from a proton donor (A) to a protein acceptor (green B). Proton exchange is mediated through hydrogen bonding which results in proton transfer. (B) Base (OH\(^-\) or OD\(^-\)) catalyzed mechanism of hydrogen exchange. Either the OH\(^-\) or OD\(^-\) anions form a hydrogen bonded complex with a backbone amide proton which leads to proton abstraction and formation of the imidate anion. The imidate anion is then re-protonated or deuterated from solvent H\(_2\)O or D\(_2\)O molecules regenerating catalyst. Adapted from (Morgan, 2011).
because of the large pKa values for the C-H bond. pKa values in the 30-50 range are common for most C-H bonds (Johnson, 1999). The differences in $k$ described above illustrate how the exchange of amide hydrogens allow amide positions to be measured using HX.

The proton transfer reaction between hydrogen atoms from solvent molecules and the protein amide positions can occur by two mechanisms (Brier, 2008). These mechanisms are base-catalyzed and acid-catalyzed proton transfer [the acid-catalyzed mechanism will not be discussed, for review see (Brier, 2008)]. The base-catalyzed reaction predominates under physiological conditions and is shown in Figure 2.11B. The reaction starts when a hydroxide anion from the solvent abstracts an amide proton. Proton abstraction leads to the imidate anion, which is then re-protonated (or deuterated) by solvent H2O molecules leading to a successful proton transfer. In amide hydrogen exchange reactions the amount of D2O is in vast excess forcing the labeling with deuterium.

2.C.3 Influence of pH, temperature, hydrogen bonding and solvent accessibility

The four factors that dictate the rate of amide hydrogen exchange are pH, temperature, hydrogen bonding and solvent accessibility. Since pH and temperature can be controlled experimentally, the deuteration of a protein in solution largely depends on hydrogen bonding and solvent accessibility. These are indicative of protein conformation and dynamics. The pH and temperature of an HX experiment are controlled to ensure efficient labeling during the reaction and retention of the label during analysis.

One of the most important factors concerning the HX reaction is pH. The work of Bai et al in the 1990’s, which studied the pH dependency of HX in poly-DL-alanine (PDLA) model
peptides by NMR, showed that the exchange rate in proteins largely depends on pH and can be explained using Equation 2.2 (Woodward, 1980; Englander, 1983; Bai, 1993; Brier, 2008).

\[ k_{ex} = (k_{H_3O^+}[H_3O^+]) + (k_{OH^-}[OH^-]) + k_{water} \]  

(2.2)

The rate of exchange \( k_{ex} \) depends on the sum of acid (\( k_{H_3O^+} \)), base (\( k_{OH^-} \)) and water (\( k_{water} \)) catalyzed proton transfer contributions. Bai et al determined the rate constants for acid, base, and water catalyzed proton exchange to be 41.7 M\(^{-1}\) min\(^{-1}\), 1.12 \( \times \) 10\(^{10} \) M\(^{-1}\) min\(^{-1}\), and 3.16 \( \times \) 10\(^{-2} \) M\(^{-1}\) min\(^{-1}\), respectively at 20 °C at low salt concentrations (Bai, 1993). If the HX exchange rate (\( k_{ex} \)) is plotted as a function of pH (Figure 2.12A) it produces a V-shaped curve where the minimum of the curve represents the slowest exchange rate. The pH range for the lowest exchange rate values are between 2.5 and 3 (highlighted by the red bar). The difference between the HX exchange rates under typical labeling conditions (physiological pH, green bar) and quench conditions is approximately three orders of magnitude. When conducting amide HX exchange, it is essential to adjust the pH of the reaction mixture after labeling to ensure retention of the deuterium label during LC-MS analysis.

Temperature is another determining factor when considering the amide exchange rate. The influence of temperature on the exchange rate can be estimated using the Arrhenius Equation 2.3 (Bai, 1993).

\[ k(x)_T = k(x)_{293} e^{-\left(\frac{E_a(x)\left(1\right)}{R\left(\frac{1}{T} - \frac{1}{293}\right)}\right)} \]  

(2.3)

The amide exchange rate constant \( k(x)_T \) for acid, base, and water catalyzed exchange at different temperatures is estimated using the rate constants for \( k_{H_3O^+}, k_{OH^-} \), and \( k_{water} \) (described in pH section), the gas constant R (8.134 J mol\(^{-1}\) K\(^{-1}\)), the apparent activation energy for acid (\( E_a \) (H\(_3\)O\(^+\)) 14 kcal mol\(^{-1}\)), base (\( E_a \) (OH\(^-\)) 17 kcal mol\(^{-1}\)) and water (\( E_a \) (H\(_2\)O) 19 kcal mol\(^{-1}\) and
Figure 2.12 Temperature and pH dependence of amide hydrogen exchange. (A) pH dependence of amide hydrogen exchange. The exchange rate is plotted vs. pH which results in the typical V shaped curve (Brier, 2008). The exchange rate for typical labeling conditions is highlighted in green. The reaction is slowed to a minimum at pH 2.5 (quench conditions, red). (B) Temperature dependence of amide hydrogen exchange. The exchange rate is plotted as a function of temperature at pH 2.5 (Morgan, 2011). (C) Amide hydrogen exchange rate as a function of different pH and temperature conditions.
temperature (T) (Bai, 1993). The HX exchange rate for different temperatures at pH 2.5 is shown in Figure 2.12B. The reduction in temperature from 25 °C to 0 °C lowers the HX exchange rate approximately 10 fold, which allows for an additional adjustment of amide exchange rate during HX MS experiments. The change in HX exchange rate as a result of a drop in temperature can be attributed to several factors including altering the concentrations of OH⁻ anions in solution as well as altering the ionization constant of water (Covington, 1966; Englander, 1972; Englander, 1983).

In order to retain the majority of deuterium label during an HX MS experiment, the combination of controlling the temperature and pH lowers the rate of amide exchange to a minimum. The effect of temperature and pH adjustment is illustrated in Figure 2.12C where the HX exchange rate is shown for different pH and temperature conditions. By lowering the pH of the reaction from 7.0 to 2.5, a reduction in exchange rate by approximately three orders of magnitude occurs. Additionally dropping the temperature of that same reaction mixture to 0 °C, results in an additional decrease in exchange rate by one order of magnitude. The minimum exchange rate achieved by altering the pH and temperature is termed quench conditions. Quench conditions are maintained during all LC-MS analysis steps.

The rate and location of exchange is dictated by hydrogen bonding and solvent accessibility. Amide hydrogens of linear fully solvent exposed peptides and proteins at pH 7 exchange very rapidly with rates of 10-1000 sec⁻¹ (Bai, 1993). HX exchange rates differ drastically in folded proteins depending on whether or not amide hydrogens are located in the interior of the protein and/or if they are involved in intramolecular hydrogen bonding. Figure 2.13 illustrates how solvent accessibility and hydrogen bonding relate to hydrogen exchange in proteins. Regions that are highly “dynamic”, solvent exposed, and/or not involved in protein
Dynamic and exposed amide hydrogens exchange rapidly. Protected amide hydrogens exchange slower after 1 minute. Hydrogen deuterium exchange after 2 hours.

Figure 2.13 Amide hydrogen exchange in proteins. Backbone amide hydrogen positions (blue) in proteins are in constant exchange with hydrogens from solvent. Once placed in D$_2$O, these backbone amide hydrogens become deuterated (red). Amide positions that are in “dynamic” and exposed regions will become deuterated rapidly while amide positions in “rigid” and solvent excluded regions will exchange more slowly. Adapted from (Marcisin, 2010).
structure hydrogen bonding (like the loops connecting the alpha helices) become deuterated rapidly while regions that are “rigid”, solvent occluded, and/or involved in hydrogen bonding networks (such as β-sheets or α-helices) will exchange more slowly. It has been shown that in folded proteins some amide hydrogens exchange quickly (sec) while others exchange on timescales from minutes to months (Englander, 1983; Smith, 1997).

2.C.4 Hydrogen exchange in folded proteins

The amide hydrogen exchange measured for folded proteins in solution is thought to occur by two distinct mechanisms, as illustrated in Figure 2.14. The exchange can occur directly into the folded form of the protein (Figure 2.14A and Equation 2.4), or exchange can occur into a partially unfolded form of the protein, as illustrated in Figure 2.14B and Equation 2.5 (Woodward, 1971; Woodward, 1980; Kim, 1993).

\[
\begin{align*}
    &F_{\text{H}} \xrightarrow{k_{\text{ex}}} F_{\text{D}} \\
    &F_{\text{H}} \xrightarrow{k_1} U_{\text{H}} \xrightarrow{k_{\text{ex}}} U_{\text{D}} \xrightarrow{k_1} F_{\text{D}}
\end{align*}
\]

The F and U terms represent the folded and unfolded forms of the protein while \(k_1, k_{-1}\), and \(k_{\text{ex}}\) are the rate constants for protein unfolding, refolding, and HX exchange, respectively. For folded proteins in solution, the observed HX exchange rate constant \(k_{\text{ex, observed}}\) is a result of contributions of both types of exchange shown above and is expressed by Equation 2.6 (Woodward, 1980; Kim, 1993).

\[
k_{\text{ex, observed}} = k_F + k_U
\]

Exchange directly into folded proteins \(k_F\) is suggested to occur by several mechanisms including: (1) the solvent penetration model, and (2) relayed imidic model (Rosenberg, 1970;
Figure 2.14 Models of hydrogen exchange in proteins. (A) Amide hydrogen exchange into the folded state (F) of a protein. $k_{ex}$ represents the amide hydrogen exchange rate constant. (B) Exchange into unfolded (U) and/or partially unfolded forms of a protein. The subscripts H and D represent the protonated or deuterated protein forms. $k_1$ and $k_{-1}$ are the unfolding and refolding rate constants respectively. Adapted from (Wales, 2006.)
Tüchsen, 1985; Tüchsen, 1987). The solvent penetration model suggests that amide hydrogens near the surface of proteins exchange with ease while buried amide positions exchange due to presence of catalyst (OH⁻ or H₂O⁺) as a result of solvent channels. The imidic model suggests that amide exchange of buried positions occurs due to a charge delocalization process. Despite the differences in the two models, one common factor required for both explanations is the small atomic movements in proteins to allow catalysts to access exchange sites.

The majority of proteins in solution undergo many rapid localized unfolding and refolding events before they are labeled with deuterium, (Equation 2.5), where the rate of protein refolding is much faster than the amide exchange rate \( k_1 \gg k_{ex} \). In this situation, a gradual increase in protein deuterium incorporation over time is observed which is defined as the EX2 exchange kinetic limit (Hvidt, 1966) as illustrated in Figure 2.15A. An alternate scenario can exist for a select number of proteins where the rate of refolding is slower than that of exchange \( k_1 \ll k_{ex} \). A large portion of the unfolded protein becomes labeled after one unfolding event which is defined as the EX1 exchange kinetic limit (Hvidt, 1966) and is illustrated in Figure 2.15B. EX1 exchange kinetics usually present multiple populations in mass spectra over deuteration time corresponding to the folded and unfolded populations. The determination of EX1 with mass spectrometry can be done by monitoring protein peak width of a protein charge state where EX1 exchange is apparent during deuteration (Weis, 2006). This can be useful in protein conformational studies and will be further discussed in section 2.D of this chapter.

2.D. Initiating hydrogen exchange and monitoring with mass spectrometry

Hydrogen exchange can be conducted once the desired protein system has been produced in quantities suitable for MS analysis. Proteins are prepared in MS compatible H₂O buffers at
Figure 2.15 EX1 and EX2 exchange patterns in proteins. (A) The EX2 regime, typical for the majority of proteins, results in a gradual mass increase over deuteration time. Peak width analysis of EX2 exchange events results in relatively constant peak widths as indicated by the flat line (Weis, 2006). (B) A small number of proteins undergo EX1 kinetics which results in the appearance of multiple mass distributions corresponding to the folded state (blue) and unfolded state (red). Peak width analysis of EX1 exchange events result in sharp peak which corresponds to the unfolding half life ($t_{1/2}$) (Weis, 2006).
near physiological pH. HX is initiated by diluting the protein samples into a D$_2$O buffer with the same buffer composition as the H$_2$O based buffer. Protein samples are then allowed to incubate in D$_2$O for the desired deuteration time before quenching the reaction.

Differences in the physical and chemical properties between hydrogen (\(^1\)H) and deuterium (\(^2\)H) allow several biophysical techniques to distinguish between the two isotopes. When the deuterium incorporation into a protein is monitored with NMR, the amide proton peaks disappear upon deuteration as deuterium is \(^1\)H-NMR silent. Another difference between hydrogen and deuterium that makes measurements of HX possible is the difference in mass. The mass of hydrogen is 1.0078 Da and deuterium 2.0141 Da. As a protein becomes deuterated, it will have a larger mass than a non-deuterated protein (Katta, 1991). Deuteration can be monitored using Fourier transform infrared spectroscopy (FTIR) as the mass increase accompanied by replacement of hydrogen for deuterium alters the vibrational frequencies of the backbone amide bond resulting in a shift at the Amide I and II regions respectively (Baenziger, 1995; Gallagher; Vagano, 2004). Mass spectrometry is another method that can easily monitor an increase in mass as a result of deuteration.

2.D.1 HX MS data acquisition and analysis

Once a protein has been labeled with deuterium and the reaction quenched (section 2.C of the HX MS workflow), the protein can be analyzed using mass spectrometry. For the work presented in future chapters, intact HX MS analysis of proteins was conducted by injecting samples into the valve setup, as illustrated in Figure 2.16A (i), through an Alltech inline analytical guard column packed with Porous 20 R2 (Applied Biosystems, Carlsbad, CA) media. Salt was manually removed from samples by washing with 1 mL of water supplemented with
0.05% TFA (HPLC buffer A) which were then eluted using a 15-98% acetonitrile gradient over five minutes (Figure 2.16A (ii)) and the eluent directed into a time of flight (ToF) mass spectrometer. A typical total ion chromatogram of HPLC eluent is shown in Figure 2.17 (i). The large peak in the chromatogram typically represents the protein of interest. The mass spectrum corresponding to the peak in (i) is shown in Figure 2.17A (ii). If the mass spectrum of the protein is magnified, the individual charge states for the protein are revealed (Figure 2.17A (iii)). From the mass/charge (m/z) value and charge state of the peak, the mass of the protein can be determined (as described below). One way to illustrate deuterium incorporation into proteins is to follow one charge state of a protein during the course of a hydrogen exchange experiment (Figure 2.18A). Detailed experimental methods for intact mass analysis can be found in appendices II and III.

Pepsin digestion post deuterium labeling was used to determine the location of deuterium incorporation into different sections of a protein (Zhang, 1993; Cravello, 2003). Pepsin digestion occurred after all the conformational information had been captured in the labeling experiment. Protein samples were quenched, passed through a column packed with immobilized pepsin on porous media to digest the protein (Wang, 2002). The typical valve setup for pepsin digestion is illustrated in Figure 2.16B (i). The resulting peptide mixture was separated using a C$_8$ or C$_{18}$ analytical separation column connected to a HPLC/UPLC. Peptides were eluted from the revered phase analytical column using an 8-98% acetonitrile gradient over 12 minutes, as shown in Figure 2.16B (ii), and the eluent directed into a Q-ToF mass spectrometer. The typical chromatogram produced by detection of all ions reaching the detector of the mass spectrometer is shown in Figure 2.17B (i). Each peak in the chromatogram contained multiple peptides as evident by the mass spectrum in Figure 2.17B (ii). Each peak in (ii)
Figure 2.16 Liquid chromatography valve systems for protein analysis. (A) (i) For intact protein analysis, proteins were injected onto a porous 20 R2 protein trap through a Rheodyne 8125 valve. The red lines are provided to show the flow paths under sample loading and sample elution conditions. Protein samples are de-salted and subsequently eluted using a 15-98% acetonitrile gradient over five minutes (ii). (B) (i) Multi-valve setup for peptic peptide analysis. Protein samples are loaded into a sample loop and then allowed to pass through a column containing immobilized pepsin which generates peptic peptides. Peptides are then passed through a Rheodyne 7000 switching valve where trapping and separation occurs using a peptide trap and analytical separation column. (ii) Peptide samples are de-salted and subsequently eluted using a 8-98% acetonitrile gradient over 12 minutes.
Figure 2.17 Mass analysis of proteins and peptides. (A) (i) Total ion chromatogram (TIC) of intact eluent. (ii) Protein mass spectrum corresponding to the combined data (red highlighted region) of the TIC trace. (iii) Magnification of the +26 charge state of the protein in (ii). (B) (i) Total ion chromatogram of digestion. (ii) The mass spectrum corresponding to the red highlighted region has multiple peptide ions that correspond to different peptic fragments. (iii) Further magnification of the red highlighted region in (ii) reveals the +4 ion of a peptide.
corresponded to a single charge state of a peptic peptide. When the mass spectrum is focused on one specific peak (Figure 2.17B (iii)), the individual charge state for that peptide is observed. Following one charge state of a peptic peptide during the course of a hydrogen exchange experiment is one way to visualize deuteration. The identity of each peptic peptide was confirmed using exact mass analysis and MS$^E$ (Silva, 2006) identification methods. Detailed experimental methods for peptide analysis are provided in appendices II and III.

After a protein of interest was incubated with deuterium for specific amounts of time (seconds-hours) and either mass analyzed as intact protein or peptides, the relative deuterium incorporation was then calculated. Determining the relative deuterium level for intact proteins and peptides is illustrated in Figure 2.18. In Figure 2.18A, a single charge state for each labeling time is shown. The relative amount of deuterium that was incorporated was calculated by taking the difference between the mass of each exchange time point and the mass of the un-deuterated control. Deconvolution of intact protein mass spectra from m/z values to mass values can be obtained from each charge state using Equation 2.8 (Mann, 1989). The average protein mass can then be determined by averaging the masses obtained from each charge state.

$$\text{mass} = ((m/z) \times z) - (z \times 1.0078)$$

Mass was calculated by using the charge state of the selected peak (z) and the corresponding m/z value. 1.0078 corresponds to the mass of hydrogen. The software program Mag Tran (Zhang, 1998) was used to conduct automated mass deconvolution.

The determination of deuterium incorporation into peptic peptides was similar to intact proteins and is shown in Figure 2.18B. The peptide isotope peaks for each labeling time point of an example experiment are shown. To determine the average amount of deuteration, the centroid mass of each time point is first calculated using Equation 2.9 (Strupat, 2005).
Figure 2.18 HX MS data processing. (A) Example of the deuteration pattern for an intact protein. Shown is a single charge state over the course of a deuteration experiment. The protein mass is calculated using the centroid (green line) m/z value of all charge states. Relative deuteration is calculated by subtracting the protein mass of each deuteration time point from the undeuterated control. The location of the peak width measurements is indicated by the orange line. (B) Example of following the deuteration pattern for a peptic peptide. (C) Calculated deuterium incorporation for panel B. (D) Deuterium uptake values are then plotted into deuterium incorporation curve.
The centroid mass of a peptide is calculated using the abundance \((I_i)\), and mass \((m_i)\) of each isotope peak from a peptide. The calculated centroid masses for the peaks shown in Figure 2.18B are displayed in Figure 2.18C. The calculated relative deuterium values for peptic peptides (see above on intact mass analysis for deuterium incorporation determination) were then plotted vs. time, which are referred to as deuterium incorporation curves and are illustrated in Figure 2.18D. The custom Excel macro HX Express (Weis et al, 2006) was used to conduct the analysis depicted in Figure 2.18.

2.D.2 Error and back exchange

The error associated with HX MS measurements can be attributed to three factors including (1) sample preparation, (2) mass measurement, and (3) data processing. The error associated with sample preparation results largely from the hydrogen exchange reaction and quenching process. Deviations in pH values between different buffers and solutions can change deuterium incorporation values. To minimize sample associated error, deuterated protein samples were prepared using identical buffers/reagents and mass analyzed on the same day. To minimize mass measurement associated error, mass spectrometers were calibrated using myoglobin for intact mass analysis or the glu-fibrinogen peptide for peptide analysis. Data processing, which can result in error if centroid mass values are not properly calculated, was conducted using the software program HX express and results were verified manually. Typical average deuterium incorporation value errors for the mass measurements presented in future chapters, as a result of the sources described above, ranged from ± 2 Da for intact mass measurements and ± 0.25 Da for peptide measurements.
With any HX MS experiment there is always loss of deuterium during the LC-MS analysis. This is commonly referred to as back exchange. Typical back exchange values can range from 15-20% depending on the instrumental setup used. It is possible to correct for back exchange [see (Zhang, 1993)], however, for the majority of work presented in future chapters, deuterium levels are reported as relative and not corrected [see (Wales, 2006) for review].

2.E What can hydrogen exchange tell us

There are many different aspects of protein chemistry that can be investigated using HX MS (Figure 2.19). HX MS has the ability to shed light on topics such as protein folding, conformation, dynamics, and protein:ligand interactions [reviewed in (Engen, 2011)]. Many examples of using HX MS to probe the topics listed above exist, however one interesting advantage of HX MS is the ability to detect different populations of molecules in solution (i.e. EX1 unfolding). If multiple populations that are different structurally exist, the deuteration patterns of the different populations will be different and easily distinguished (Miranker, 1993), as illustrated in Figure 2.20. The protein shown in Figure 2.20A undergoes EX1 exchange kinetics (see Figure 2.15B) indicated by the two peaks. The approximate point where there is a 50:50 ratio of folded to unfolded species, indicated by the asterisk, corresponds to the unfolding half life. The unfolding pattern of the protein can be used to assay for protein:ligand binding as the rate of unfolding can be altered (slowed) as a result of stabilization effects from ligand binding (Figure 2.20A). The observation of changes in EX1 unfolding half lifetimes can be an invaluable tool to assay protein:ligand binding. EX1 unfolding data can enable the extraction of relative binding affinities (Trible, 2007), or determine when and if certain sequences are able to associate intramolecularly (Chen, 2008; Hodkinson, 2009).
Figure 2.19 Many uses of HX MS. HX MS can provide valuable information on protein folding (blue), conformation (orange), dynamics (tan), and protein-ligand interactions (purple) (Engen, 2011).
Figure 2.20 Utility of EX1 exchange in monitoring protein:ligand binding events. (A) The protein alone undergoes unfolding with a half life of approximately 15 minutes (indicated by the asterisk). (B) Upon ligand binding, the unfolding half life is slowed to 30 minutes. Adapted from (Marcisisin, 2010).
To further illustrate the capabilities of HX MS consider Figure 2.21, which illustrates the conformational information that can be obtained from HX MS deuteration curves. Presented are two hypothetical situations using HX MS to probe the interaction of a protein plus small molecule inhibitor as well as a protein-protein interaction. In Figure 2.21A, analysis of the intact protein (i) can reveal if there are any global conformation and/or dynamic changes as a result of inhibitor binding. In this example, the small molecule inhibitor causes a reduction in deuteration (red curve). The location of the deuteration differences can be observed (ii and iii) by digesting the deuterated protein with a protease. Residues 20-35 of this protein (ii) undergo a reduction in deuterium incorporation upon inhibitor binding while residues 70-89 (iii) do not. The location of each peptide is known (structural insets) and comparison with 3D structural information (such as X-ray or NMR structures) allows for a more complete understanding of the protein:ligand interaction. Figure 2.21B illustrates deuteration changes as a result of oligomer formation. The deuterium incorporation curves from the intact protein analysis (i) indicates protection from deuteration upon formation of the hexamer and peptide analysis reveals the location of structural changes to residues 10-28 (ii) and not residues 50-68 (iii). The information depicted in Figure 2.21B can reveal the possible location of subunit interfaces and possible allosteric conformational changes as a result of protein complex formation.

2.F Summary

HIV Vif needed to be expressed and purified before biophysical characterization could take place. HIV-1 Vif was successfully isolated from E. coli using a denaturing purification protocol followed by protein refolding as described in section 2.A. Renatured Vif produced with the denaturing protocol was not stable at concentrations above 50 μM and was extremely
Figure 2.21 Information obtained from deuterium incorporation curves. (A) Examples of deuterium incorporation curves for a protein plus small molecule inhibitor and (B) protein-protein interaction. The graphs indicate structural changes that can be observed on the intact protein level (i) as well as localization by pepsin digestion (ii and iii). Adapted from (Marcisin, 2010).
sensitive to aggregation. The aggregation tendency of Vif has prevented structural investigation by other more common biophysical techniques and was the reason why HX MS was chosen for conformational analysis. The background and theory of HX MS and information that can be obtained using the technique were presented in sections 2.C-D and provide the basis for the understanding of HX MS results described in future chapters.

2.G References:


CHAPTER 3
Functional and Conformational Analysis of HIV-1 Vif

Parts of this chapter have been published and are included in Appendix II:

Special focus issue: Structural and Molecular Biology of HIV

3.A Introduction

The use of recombinant HIV-1 Vif produced for structural investigation with the Yang et al denaturing protocol described in Chapter 2 raised the following concern: Does Vif renatured from a denaturing purification retain relevant biochemical functionality? The biochemical functionality of recombinant Vif needed to be assessed so that any conformational information obtained using HX MS would reflect a relevant conformation of Vif in solution. Figure 3.1 illustrates the production process for Vif and the different methods of assaying functionality. Proteins that are enzymes are generally tested using activity assays. Assessing the functionality of proteins that do not contain enzymatic activity (such as Vif) requires testing functionality via other readouts. The main function ascribed to Vif is to bind to cellular proteins; therefore the best assays to probe Vif functionality are binding assays that test the interaction between Vif and interacting proteins such as APOBEC3G, Cullin 5, Elongin BC, and Hck SH3. This Chapter is divided into two parts: (1) functional analysis of recombinant Vif and (2) conformational analysis of recombinant Vif. Section 3.B describes the use of an HX MS binding assay probing the solution phase interaction between Vif and the SH3 domains of the Hck, Lck, and Lyn, all members of the Src family of protein tyrosine kinases (SFKs). An in vitro kinase activation assay
Figure 3.1 Assessing the biological functionality of recombinant Vif. The process for assessing Vif functionality is outlined.
is also described that probed the Vif dependent activation of different SFKs. The conformational analysis of Vif with CD, and HX MS is also discussed.

3.B Functional analysis of recombinant Vif

3.B.1 HX MS assay for SFK SH3:Vif binding

Vif binds to an array of viral and host cellular proteins which could potentially be used to test Vif functionality. Of the different interacting proteins, Vif has been shown previously to specifically bind the human hemopoietic cell kinase (Hck), an enzyme expressed in the HIV-1 target cell macrophages. This interaction resulted in altered kinase activity which affected HIV-1 infection (Hassaine, 2001; Douaisi, 2005). Hck belongs to the Src family of non-receptor protein tyrosine kinases (SFKs). The SFKs share similar tertiary structure, domain organization, and patterns of activation (Engen, 2008) as illustrated for Hck in Figure 3.2. Hck contains a C-terminal kinase domain (blue) that is tethered to several regulatory elements (green and yellow) by a linker (red). These regulatory elements include the Src homology 3 domain (SH3, yellow) and Src homology 2 domain (SH2, green) (Roskoski, 2004).

There are several different mechanisms in which SFKs can become activated through association of activator proteins with the SH3 and SH2 regulatory elements as illustrated for the SH3 domain in Figure 3.2B. The work of Hassaine et al and Douaisi et al showed by pull-down analyses that association of Vif and Hck was mediated primarily through the poly-proline type II helix of Vif (PPII, see Figure 1.6A and 3.2B) and the SH3 domain of Hck (Hassaine, 2001; Douaisi, 2005). Besides Hck, Vif has been shown to associate with the SH3 domains of the SFKs Fyn, Lyn, and Lck (Douaisi, 2005). The ability of Vif to interact with different SFK SH3 domains and alter kinase activity provided the basis to assay Vif functionality as incubation of
Figure 3.2 Src family tyrosine kinase (SFK) structure and activation. (A) Domain organization of SFKs. The kinase domain (blue) is connected to the SH2 (green) and SH3 (yellow) regulatory domains by the linker region (red). (B) Activation of SFKs. Kinase activation usually results from binding of an activator protein (brown) to the SH3 or SH2 regulatory regions (Young, 2001; Hantschel, 2004; Engen, 2008). The example in (B) illustrates SH3 domain displacement and binding of a poly-proline type II motif (PPII) by an activator protein.
recombinant renatured Vif with the SFKs should result in binding and modulation of kinase activity.

HX MS is another method that has been used to assay SH3 binding due to the unique solution dynamics of certain SH3 domains (Engen, 1997; Wales, 2006; Trible, 2007;). The SH3 domain of Hck is one example of an SH3 domain with unique solution dynamics. Hck SH3 undergoes slow cooperative unfolding in solution on a timescale that can be monitored readily with HX MS due to the appearance of multiple populations in mass spectra as illustrated in Chapter 2, Figure 2.15. The multiple populations observed in EX1 unfolding events arise from the differential labeling of a folded more protected species and an unfolded more easily deuterated species (Wales, Mass Spectrom Rev 2006).

The Hck SH3 unfolding event can be used as an assay for protein binding because ligand binding results in changes in the unfolding half-life [illustrated in (Trible, 2007; Engen, 1997; Weis, 2006) and Figure 2.20]. The magnitude of how much a ligand slows protein unfolding is referred to as “slowdown factor” and is used to quantify the change in the protein unfolding half-life (Trible, 2006; Hochrien, 2006; Chen, 2008). To determine slowdown factor from EX1 exchange kinetics as a result of ligand binding, HX MS analysis is conducted on the protein alone (i.e. SH3 domain) and in the presence of a ligand as illustrated in Figure 3.3. Peak width measurements are made throughout the HX experiment and the unfolding half-life calculated by plotting peak width vs. time. The apex of the peak width vs. time plot corresponds to the protein unfolding half-life ($t_{1/2}$) as shown in Figure 3.4. When a ligand binds to a protein that undergoes EX1 exchange kinetics, a shift in the unfolding half-life can occur due to conformational stabilization (illustrated in Chapter 2, Figure 2.20). The unfolding half-life for the protein alone ($t_{1/2, \text{reference}}$) and the unfolding half-life for the ligand bound protein ($t_{1/2, \text{test}}$) are entered into
Figure 3.3 Vif:SH3 HX MS binding assay. To assay Vif binding to the different Src family tyrosine kinase domains, SFK SH3 domains were labeled with deuterium in the presence and absence of Vif (blue section). SH3 domains were mass analyzed (green section) and the unfolding half life determined in the presence (t_{1/2,test}) and absence (t_{1/2,reference}) of Vif (red section).
Figure 3.4 Determining slowdown factors for EX1 unfolding. The unfolding half life ($t_{1/2}$) of an EX1 unfolding events can be used to determine the slowdown factor as a result of protein:ligand binding (see Figure 2.20). $t_{1/2,reference}$ represents the unfolding half-life of the unbound protein and $t_{1/2,test}$ represents the unfolding half-life of the bound protein. Slowdown factors are normalized to the protein unfolding half-life of the unbound protein. Adapted from (Chen, 2008)
Equation 3.1 to determine the slowdown factor as a result of ligand binding as illustrated in Figure 3.4 (Chen, 2008).

\[
\text{Slowdown Factor} = \frac{t_{1/2, \text{test}}}{t_{1/2, \text{reference}}} \tag{3.1}
\]

Slowdown factor analysis is useful because it can give insight into binding affinities and also be used to compare different proteins and protein constructs (i.e. SH3 domains) (Hochrein, 2006).

The SH3 domains of different SFKs needed to be obtained before HX MS testing of Vif binding could occur. The Lyn, Lck, and Hck SH3 domains were chosen for testing with Vif. There were several reasons for choosing these SFK family members. Hck was the primary kinase chosen because of the documented association with Vif (Hassaine, 2001; Douaisi, 2005); Lyn and Lck were chosen because these kinases (like Hck SH3) display EX1 unfolding in solution, as not all SFK SH3 domains undergo EX1 exchange (Wales, 2006). The reason for choosing Lck was due to its role in T-cell signaling and possible interaction with Vif. The Lyn kinase SH3 domain was chosen due to Lyn’s dynamic similarity to Hck, and the presence of Lyn in the HIV-1 target cells macrophages. The Lyn and Lck SH3 domains were obtained from Thomas Wales (Northeastern University) and the Hck SH3 domain was expressed and purified from \textit{E. coli} (Wales, 2006; Poe, 2009). As a negative control of binding the inactivating W93A Hck SH3 variant was produced. Mutation of tryptophan93 to alanine abolishes Hck SH3:Vif binding (Hassaine, 2001; Poe, 2009). All SH3 domains used to assay Vif binding were checked with ESI-MS to verify the correct mass as shown in Figure 3.5. The recombinant SFK SH3 domains described in this Chapter and the recombinant HIV-1 Vif described in Chapter 2 were then subjected to HX MS analysis as illustrated in Figure 3.3.
Figure 3.5 ESI-mass spectra of SH3 domains used for HX Vif binding assay. The charge state distributions of the SH3 domains used to assay Vif binding are shown along with the deconvoluted mass.
3.B.2 HX MS binding assay results

The binding between Vif and the different SFK SH3 domains was tested with HX MS and results for each SH3 domain are shown in panels A-D of Figure 3.6. Each panel corresponds to one of the SH3 domains tested. The raw spectra are shown in (i); deuterium incorporation in (ii); and peak width analysis in (iii). The HX MS results for the Hck SH3 domain, shown in Figure 3.6, provide information on the unfolding half-lives in the raw MS spectra by the wide m/z distribution indicated by the asterisk. The unfolding half-life for Hck SH3 alone is at 30 minutes, and 2 hours in the presence of Vif. Deuteration for Hck SH3 was decreased upon incubation with Vif as shown in the deuterium incorporation graph in Figure 3.6A (ii). The change in deuteration and corresponding shift in the unfolding half-life due to the presence of Vif suggests that Vif interacted with and altered the solution dynamics of Hck SH3.

The wt Hck SH3:Vif interaction was compared to that of the Hck W93A SH3:Vif interaction. The unfolding half-life for Hck SH3 W93A alone occurred at approximately 3 minutes in the absence of Vif and at 5 minutes in the presence of Vif. The change in unfolding half-lives between wt Hck SH3 and Hck W93A can likely be attributed to loss of stabilizing packing effects resulting from substitution of the more bulky tryptophan amino acid to alanine. Deuteration for Hck SH3 W9A was not altered upon incubation with Vif as shown in the deuterium incorporation graph in Figure 3.6B (ii). The deuterium incorporation curves for Hck W93A have two separate discontinuous lines (instead of one continuous line as for wt Hck SH3) which correspond to the deuteration of the folded and unfolded species. This was done for W93A because the two populations were easily observed and measurements could be made on both populations. The lack of shift in unfolding half-life or change in deuteration indicated that Vif
**Figure 3.6 Vif:SH3 HX MS results.** Shown in each panel are the raw HX MS Vif binding data (i) for Hck SH3 (A), Hck SH3 W93A (B), Lck SH3 (C), and Lyn SH3 (D). The charge state of the ion shown is indicated. The grey horizontal line shown on each peak is provided as a visual reference to gauge peak width. The approximate unfolding half life for each SH3 domain (except for Lck SH3) is indicated by the red asterisks. The deuterium incorporation graphs for each SH3 domain are shown in (ii) and peak width plots in (iii). The error of intact HX MS was ± 2 Da. Adapted from (Marcisin, 2011).
did not associate with Hck W93A *in vitro*. These results indicate that the wt Hck SH3:Vif interaction was specific as detected by HX MS.

The results for Lck SH3 are shown Figure 3.6C. The work of Weis *et al* indicated that Lck SH3 underwent rapid EX1 exchange kinetics on the timescale of seconds (Weis and Kjellen, 2006). In comparison, the results for Lck SH3 in panel C indicate that under the experimental conditions used, EX1 exchange kinetics were not clearly observed as described below. These observations can be explained by the different experimental conditions used in the work presented and that described in (Weis and Kjellen, 2006). The buffers used in the work presented in Figure 3.6 contained glycerol and higher salt concentrations than those used in the work of Weis *et al* and Wales *et al* (Weis and Kjellen, 2006; Wales, 2006). The addition of glycerol and higher salt concentrations likely altered the solution dynamics of Lck SH3. These experimental differences also explain the difference in values for unfolding half-life of the Hck domain shown in Figure 3.6A (~30 minutes) and that described in Wales *et al* (~19 minutes) (Wales, 2006). The peak width analysis of Lck SH3 did not provide insight to whether or not Lck SH3 associated with Vif, however, deuterium incorporation did. Deuteration in Lck SH3 alone was greater than when incubated with Vif. The observed decrease in deuteration of Lck SH3 upon Vif incubation indicates that Vif associated with Lck SH3 in solution.

HX MS results for the Lyn SH3 domain are shown in Figure 3.6D. The work of Wales *et al* demonstrated that Lyn SH3 has similar solution dynamics to Hck SH3 (Wales, 2006). The results in Figure 3.6D (i) for Lyn SH3 indicate that the unfolding half-life in the absence of Vif was approximately 30 minutes and similar to Hck SH3 as indicated by the asterisk. However, unlike the Hck SH3 domain, the unfolding half-life and deuteration of Lyn SH3 was not altered by the presence of Vif. Several possibilities could explain these observations. The first
possibility was that the Lyn SH3 domain did not associate with Vif in solution. The second possibility was that Vif binding did not alter the deuteration or unfolding half-life of Lyn SH3. Results presented below in Section 3.B.3 of Chapter 3 support the latter explanation.

The unfolding half-life values for each SH3 domain exhibiting EX1 exchange kinetics determined from the results in Figure 3.6(iii) were then used to determine slow-down factors as a result of incubation with Vif (Figure 3.7). Vif incubation slowed unfolding of wt Hck SH3 domain by a factor of 4.1 which is indicative of binding (Hochrein, 2006). Slow-down factor analysis of Hck W93A and Lyn SH3 indicated that incubation with Vif did not alter the unfolding half-life for these SH3 domains. The errors reported in Figure 3.7 are from duplicate slow-down factor determinations from duplicate experiments.

3.B.3 In vitro kinase activation assay

The ability of Vif to associate with Hck, and Lck SH3 domains in vitro as detected with HX MS was an interesting observation and raised the question of whether recombinant Vif could alter SFK activity. In vitro activation of different SFKs by recombinant Vif was probed using the Z’-lyte™ kinase assay. The workflow for the Z’-lyte assay is shown in Figure 3.8. For the Z’-lyte assay, a down-regulated form of each SFK was incubated with a potential activator protein (such as Vif) and a phosphorylatable substrate peptide labeled with coumarin and fluorescein. The activation of the SFK by the activator protein would result in phosphorylation of the substrate peptide. The lack of activation would result in unmodified peptide. The kinase reaction was quenched and the mixture developed with a reagent that cleaved any non-phosphorylated peptide. The developed reaction mixture was finally subjected to fluorescence analysis. The presence of phosphorylated intact peptide resulted in fluorescence resonance energy transfer
Figure 3.7 Slowdown factor analysis for Vif:SH3 binding. Shown are the slowdown factor results for Hck (red), Hck W93A (grey) and Lyn SH3 (green) for Vif binding. Slowdown factors were determined as described in Figure 3.3. The error of SF measurements was ± 0.2 as calculated from duplicate slowdown factor determinations. Adapted from (Marcisisin, 2011).
Figure 3.8 Overview of the Z’-lyte kinase assay. The four steps of the Z-lyte assay are depicted. Step 1 consists of incubating a Src family tyrosine kinase with a potential activator protein. Step 2 is the actual kinase reaction. Step 3 is the development and step 4 is detection. There is an increase in % phosphorylation (red bars) of the substrate if the activator protein activates the Src family tyrosine kinase. The blue bars represent a lack of kinase activation. Adapted from Z’-lyte product manual (Invitrogen, Carlsbad, CA).
(FRET), while cleaved peptide resulted in Coumarin fluorescence. The fluorescence emission ratio of Coumarin to FRET (445/520 nm) was used to determine the percent phosphorylation of the substrate peptide which is indicative of SFK kinase activity. Results from the Z’-lyte assay are shown in Figure 3.9 for Hck, Lck, and Lyn which suggest that incubation of SFKs with increasing amounts of recombinant full-length Vif resulted in increased kinase activity. These data indicate that recombinant Vif interacted with and activated Hck, Lck, and Lyn in vitro. The Z’-lyte activation results are consistent with HX MS binding experiments for Hck and Lck. The Lyn kinase was activated by incubation with recombinant Vif as illustrated in Figure 3.9; however, Vif incubation with the Lyn SH3 domain did not result in altered SH3 solution dynamics. This observation can likely be explained by the possibility of Vif interacting with Lyn SH3 differently than Hck SH3 in a way that did not alter SH3 unfolding. Alternatively, Vif could be interacting with another region (e.g. SH2) of Lyn resulting in kinase activation. This explanation is consistent with the observation that Vif activated Lyn, but failed to alter Lyn SH3 solution dynamics. HIV-1 Vif was also tested for in vivo SFK activation in yeast cells at the University of Pittsburg by Purushottam Shivaji (data not shown, see appendix I). In vivo activation results were similar to the in vitro experiments described above as HIV-1 Vif co-expressed in yeast with Lck, Lyn, and Hck resulted in kinase activation.

The in vitro binding experiments between Vif and Hck SH3 and the Z’-Lyte kinase activation assay results are consistent with previous reports that Vif interacted with Hck. In addition to Hck, the SFKs Lyn and Lck were tested for interaction with Vif. The studies presented above suggest Vif is a potent activator of the SFK’s. These studies also support the idea that Vif expressed and purified under denaturing conditions adopts a biochemically functional conformation in context to Hck SH3 binding and kinase activation. Further evidence
Figure 3.9 Vif activation of Src family tyrosine kinases. Near full-length Hck, Lyn, and Lck were combined with Vif at the molar ratios shown, and kinase assays were performed using the FRET-based Z’-Lyte assay (see Figure 3.8). Data were acquired with the assistance of Lori Emert-Sedlak (University of Pittsburgh). Adapted from (Marcsisin, 2011).
indicating the biochemical functionality of Vif are the reports that full-length recombinant Vif specifically interacted with the Elongin BC complex [described in Chapter 4 (Marcisin, 2010)] and DNA/RNA oligonucleotides (Bernacchi, 2007; Mercenne, 2010).

3.C Conformational analysis of recombinant Vif

3.C.1 Circular dichroism analysis of Vif

Having established that Vif produced recombinantly retained a functional conformation; the recombinant protein was then subjected to biophysical analysis. To quickly determine if Vif contained any secondary structure, renatured Vif was analyzed by circular dichroism (CD). The CD results are shown in Figure 3.10A. Two different samples were analyzed with CD; the renatured material and Vif in 8M guanidine hydrochloride (GdnHCl). The CD trace for renatured Vif (red) shows a minimum around 215 nm and subsequent analysis of the CD spectrum with the software program CDPro (Sreerama, 2000) (Figure 3.10B) suggested that much of Vif was unstructured, with some secondary structure present as β-sheet. The spectrum of Vif in 8 M guanidine hydrochloride (blue) showed a minimum around 210 nm and subsequent CDPro analysis indicated that the secondary structure distribution relative to renatured Vif shifted to less alpha helical content. These results indicate that the conformation of Vif in GdnHCl differs from the renatured form. The CD results for renatured Vif are consistent with the work of Gallerano et al in which they also determined that the majority of secondary structural elements in recombinant renatured Vif were β-sheet (Gallerano, 2010).
Figure 3.10 Circular dichroism analysis of Vif. (A) Circular dichroism spectra of recombinant Vif in native buffer (red) and in 8M GdnHCl (blue). (B) Secondary structure calculations from the Vif CD spectra in panel B, as determined with the CDPro algorithm (Sreerama, 2000). Adapted from (Marcisisin, 2011).
3.C.2 Intact HX analysis of Vif

Having established the functionality of Vif with SFK binding and probing the secondary structure via circular dichroism, the conformation and dynamics of Vif were further investigated using HX MS. The conformation of Vif alone was studied and compared to its conformation in the presence of Zn$^{+2}$. Vif is a zinc binding protein and the coordination of Zn$^{+2}$ by the novel HCCH zinc finger is thought to result in a conformational change that exposes key residues required for interaction with the E3 ligase component Cullin 5 (Xiao, 2005). The global HX MS results for Vif are shown in Figure 3.11. Shown in Figure 3.11A and B is the +26 charge state for Vif in the absence (A) or presence (B) of Zn$^{+2}$. The isotope distribution for the +26 charge state of Vif in the absence of Zn$^{+2}$ shifted to the right of the dotted line (fixed at m/z 870) after only 10 seconds in D$_2$O. This is in contrast to Vif incubated with Zn$^{+2}$ which took more than 5 minutes to cross the line. The change in deuterium incorporation is apparent in the deuterium uptake curves shown in Figure 3.11C. The global HX MS analyses for Vif alone and in the presence of Zn$^{+2}$ provides insight into the solution conformation of Vif.

The deuteration profiles in Figure 3.11C give a relative indication as to how much of Vif was highly exposed/not structured versus highly protected/structured. Vif contains 181 exchangeable backbone amide hydrogen positions and only 110 of these positions became deuterated after 2 hours of exchange in both Vif alone and in the presence of Zn$^{+2}$. The deuteration values reported in Figure 3.11 are reported as relative deuterium incorporation (see Chapter 2.C.2), as there was no correction for back exchange. Taking into account the possibility of 15-20% back exchange, deuteration values could have been in the range of 130-138 deuterons. Even with no back exchange considerations, there were 43-51 backbone amide hydrogens in Vif that did not exchange after 2 hours in D$_2$O. The protection from deuteration in
Figure 3.11 Intact HX MS results for HIV-1 Vif. (A) The +26 charge state of Vif as it incubated with deuterium with time. (B) shows the +26 charge state of Vif in the presence of Zn^{2+}. The deuterium incorporation into Vif is suppressed in the initial time points and this difference becomes less apparent with longer incubation times. The dotted lines are provided as a visual reference. (C) Deuterium incorporation plot for Vif in the absence (black line) or presence of (dotted line) Zn^{2+}. The error of intact HX MS measurements was ± 2 Da. Adapted from (Marcisisin, 2011).
Vif indicates that one or more regions of the protein backbone contained elements that hindered deuteration. As described in Section 2.B.3, the factors that could contribute to observed amide protection are hydrogen bonding and solvent occlusion. The presence of secondary structural elements (indicated by CD analysis, see Section 3.C.1), oligomeric complexes, and/or burying of amide positions in the core of the protein would explain why Vif does not completely exchange all amide hydrogens after 2 hours. These results are consistent with the CD analysis of Vif and suggest that structured elements were present.

In addition to the information about protection from exchange after 2 hours of labeling, deuteration for short times is also insightful. Amide hydrogens that exchange within 10 seconds are highly solvent exposed and not involved in hydrogen bonding networks (Dharmasiri, 1996; Truhlar, 2006). For Vif in the absence of Zn$^{+2}$, there were approximately 85-105 amide positions that became deuterated after 10 seconds. The higher number (105) takes into account back exchange. The high number of backbone amide positions that exchange rapidly suggested that unstructured and highly solvent exposed regions were present. Vif incubated with Zn$^{+2}$ showed protection from deuteration when compared to Vif alone. The difference in deuteration between Vif alone and Vif incubated with Zn$^{+2}$ was approximately 20 Da. This difference between the free and zinc-bound Vif became smaller and smaller over time and eventually both forms became equally deuterated by the 2 hour time point. The deuteration results for Vif are consistent with proteins that are protected initially from exchange due to decreases in solvent accessibility as a result of structure formation, but, are still dynamic in solution. Eventually over time the deuteration values become similar, this concept is reviewed in (Morgan, 2009). The results from intact HX MS analysis of Vif are consistent with the work of Giri et al which probed the Zn$^{+2}$ binding properties of synthetic portions of the zinc binding region (Giri, 2009;
Giri and Maynard, 2009). Giri *et al* found that the Vif zinc binding region underwent a conformational change as a result of zinc coordination. The HX MS results described above indicated that when full length recombinant Vif coordinated Zn$^{+2}$, structural changes occurred similar to those described for short fragments of Vif. The conformational changes detected with HX MS might be important for association with the E3 ligase component Cullin 5 as the Vif Zn$^{+2}$ binding region is responsible for this interaction (Xaio, 2007)

3.C.3 Peptide HX analysis of Vif

The results from intact HX MS analysis of Vif were insightful and showed that a portion(s) of Vif was highly protected from exchange. To determine the location of these protected elements pepsin digestion was utilized post deuterium labeling. The Vif peptic peptides generated using the scheme shown in Chapter 2 Figure 2.16B are shown in Table 3.1. A total of twelve peptides were identified that were used to follow Vif deuteration. The deuterium incorporation graphs for each Vif peptide are shown in Figure 3.12. Plotted in the deuterium incorporation graphs is the average deuteration values from duplicate experiments for Vif alone (blue line) and Vif incubated with Zn$^{+2}$ (pink line). Three deuterium uptake graphs are highlighted (light blue, green, and orange) to illustrate the different types of deuteration in Vif. The light blue highlighted graph shows a portion of Vif that displayed protection from exchange as deuteration levels are similar for the 10 second and two hours labeling times. The green highlighted graph shows a dynamic portion of Vif that was initially protected from exchange and eventually became deuterated over time. The orange highlighted graph illustrates a portion of Vif that was highly solvent exposed and became rapidly deuterated. To better visualize deuteration into the different regions of Vif, a deuteration map was constructed and is shown in Figure 3.13.
Table 3.1 Peptic peptides of HIV-1 Vif. Shown is the sequence and MS information for all identified Vif peptides used for HX analysis.

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Length</th>
<th>Peptide Sequence</th>
<th>m/z and z</th>
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<td>8</td>
<td>8</td>
<td>MENRWQV</td>
<td>547.2 +2</td>
<td>7</td>
</tr>
<tr>
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<td>8</td>
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<td>523.7 +2</td>
<td>7</td>
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<tr>
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<tr>
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<td>YHIPLGDARL</td>
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<td>8</td>
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<tr>
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<tr>
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<tr>
<td>134</td>
<td>147</td>
<td>14</td>
<td>EYQAGHKVGSQY</td>
<td>797.3 +2</td>
<td>13</td>
</tr>
<tr>
<td>151</td>
<td>169</td>
<td>19</td>
<td>AALITPKKIKPPLPSVTKL</td>
<td>1008.1 +2</td>
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</tr>
<tr>
<td>153</td>
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<td>13</td>
<td>LITPKKIKPPLPSVTKL</td>
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<td>12</td>
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<td>880.0 +2</td>
<td>11</td>
</tr>
<tr>
<td>170</td>
<td>192</td>
<td>22</td>
<td>TEDRWNKOPQDKHTGKHSHTMNQKL</td>
<td>676.3 +4</td>
<td>21</td>
</tr>
</tbody>
</table>
Figure 3.12 Deuterium incorporation curves for Vif peptic peptides. Relative deuterium uptake values (Da) are plotted vs. time in D₂O. The Y-axis of each graph is set to the maximum possible deuterium incorporation of each indicated peptide. The primary structure of each peptide is indicated as well as the amino acid residues. The peptide data for Vif alone (pink line) along with the peptide data for Vif with zinc (blue line). The three peptides highlighted in light blue, green, and orange are discussed in the text. The data shown are the average of two replicate experiments and the error of peptide HX MS measurements was ± 0.25 Da. Adapted from (Marcisisin, 2011).
Shown is the primary structure for Vif and the corresponding biologically relevant regions. Below the primary structure are bars that represent each of the Vif peptic peptides. The bars are color coded to their relative % deuteration which was calculated using Equation 3.2

\[
\text{Relative % Deuteration} = \frac{\text{Measured relative deuteration}}{\text{Total possible deuteration}}
\]  

(3.2)

The deuteration of the N-terminal region of Vif differed drastically from the C-terminal region. The N-terminal region displayed the least deuterium incorporation including residues 1-16 and 40-86. These regions only became 30-50% deuterated after 2 hours in D$_2$O. These highly protected regions consist of the regions in Vif necessary for APOBEC3G/F and RNA/DNA binding. Deuteration of the C-terminal half of Vif was drastically different in that most C-terminal peptides became \( \geq \) 60% deuterated after only 10 seconds in D$_2$O. The C-terminal region of Vif is responsible for interactions with multiple cellular proteins and contains the HCCH zinc finger, viral SOCS box, C-terminal APOBEC3G binding site, Hck binding site, and multimerization domain. The rapid deuteration of the C-terminal region of Vif is consistent with the idea that the C-terminal portion is unstructured in the unbound state. The work by Reingewertz et al utilized a synthetic peptide approximating residues Vif$_{145-192}$ of Vif. Using NMR and circular dichroism they showed that Vif$_{145-192}$ was unstructured in solution (Reingewertz, 2009). The peptide HX MS results for Vif presented above are consistent with the work using Vif$_{145-192}$, and are the first to show that the C-terminal portion of full-length HIV-1 Vif is unstructured in solution (Marcsisin, 2011).

Raw MS spectra for selected Vif peptic peptides are shown in Figure 3.13B-E. These representative peptides were chosen to show the deuteration patterns for different regions of Vif. Interestingly, for most of Vif, the isotopic distributions for peptic peptides were characteristic of a single population in solution and characteristic of EX2 exchange kinetics (see Chapter 2.B.4,
Figure 3.13 Peptide HX MS results for HIV-1 Vif. (A) The deuterium map for recombinant Vif. The bars are colored according to the relative percent deuterium incorporation (color code at the top) at each time in deuterium (at the left-hand side). Also indicated are the different regions of Vif and their corresponding biological functions (see also Figure 1). (B-E) Mass spectra for several Vif peptic peptides. The amino acid sequence of each peptide is shown at the top. Deuteration times are shown in each panel; UN represents undeuterated protein. The locations of the peptides in panels B-E are indicated with arrows below panel A and with the m/z of each ion. The error of peptide HX MS measurements ranged from ± 0.25 Da. Adapted from (Marcsisin, 2011).
The peptide in Figure 3.13B was initially protected from exchange and slowly becomes deuterated over time. The peptide shown in Figure 3.13E was not protected and became rapidly deuterated. Unlike the peptides shown in Figure 3.13B and E, several regions of Vif displayed multiple isotopic distributions indicative of multiple conformational states in solution (i.e. EX1 exchange kinetics). Peptides displaying EX1 exchange kinetics are shown in Figure 3.13C and D. These regions encompass portions of the N-terminal APOBEC3G binding site as well as the HCCH zinc finger. The spectra shown in Figure 3.13C and D indicate that there is conformational heterogeneity in some parts of Vif.

One of the regions in Vif that showed an interesting deuteration levels was the polyproline type II (PPII) motif encompassing residues $^{163}$PPLP$^{164}$ (Figure 3.13E). The PPII motif has been suggested to be important for several functions of Vif including SH3 binding, APOBEC3G binding, and multimerization (Wolfe, 2011; Bernacchi, 2011; Donahue, 2008; Hassaine, 2001). The PPII region was highly deuterated after only 10 seconds in D$_2$O as it exchanged nine out of eleven possible amide hydrogens. The deuteration results for the PPII helix indicate that this region was exchange competent and likely unstructured. Some researchers have suggested that the PPII region is directly involved in multimerization as a result of association of multiple Vif molecules (Bernacchi, 2010). The amide exchange observed for this region and in the C-terminus is not consistent with the idea that the PPLP domain forms the actual oligomerization interface, as the association of multiple Vif molecules would likely result in protection against deuteration. One plausible explanation for the role of the PPII motif in Vif oligomerization is that it could possibly act as a conformational hinge, bringing the necessary regions of Vif required for multimer formation together, such as the N- and C-terminals. The bridging idea is consistent with previous biochemical and chemical cross linking studies using recombinant Vif where cross links...
were observed between the N- and C-termini of Vif (Auclair, 2007). Biochemical observations have demonstrated that mutation of the prolines in the PPLP domain to alanines (AALA) decreased, not abolished, multimerization (Bernacchi, 2011). The PPLP to AALA mutation would be expected to result in more conformational flexibility as previously reported for proline to alanine substitutions (Bailey, 1990; Lin, 2005).

Another interesting observation that was made from peptide level HX experiments was the effect of Zn$^{+2}$ binding on the zinc binding region in Vif. The Vif zinc binding domain corresponds to residues H$^{108}$-C$^{114}$-C$^{133}$-H$^{139}$ (Xiao, 2006; Xiao, 2007; Xiao, 2007) and the HX results for the Vif peptic peptide (residues 115-129) encompassing this region are shown in Figure 3.14. The Vif 115-129 peptide displayed multiple conformational states in solution, apparent in the 10 second time point. The isotopic distributions that correspond to the different conformational states were fit using two Gaussian distributions with the software program Peak Fit™, Systat Software Inc (San Jose, CA). The mass distribution (blue) that was less deuterated, likely corresponds to a more folded state(s) of the HCCH zinc finger while the mass distribution (red) that was more deuterated likely corresponds to an unfolded state(s). The time required for inter-conversion between the two forms was slowed upon Zn$^{+2}$ addition. The half-life for inter-conversion of the zinc unbound form was approximately 10 seconds while the half life of the zinc bound form was shifted to approximately 1 minute as indicated by the green asterisk. The HX MS results for the Vif 115-129 peptide suggest that the Zn$^{+2}$ binding region was highly dynamic in solution and inter-converted between different conformational states. The conformational stabilization upon Zn$^{+2}$ binding in this region could be required for interaction with Cullin 5 and other components of the E3 ligase machinery.
Figure 3.14 Conformational effects of Zn\(^{+2}\) binding. Hydrogen exchange mass spectra of the Vif peptide covering residues \(^{115}\text{FSDSAIRKALLGHIV}^{129}\) in the absence (A) or presence (B) of zinc. The dotted lines are provided as a visual reference and are fixed at m/z 860 in both panels. The multiple populations present in the 10 second time point are indicated with the blue and red distributions. (C) deuterium incorporation curves for the peptide shown in panels A and B. The error of peptide HX MS measurements was ± 0.25 Da. The approximate half-lives of unfolding for Vif unbound and bound to zinc are indicated by the green asterisk. Adapted from (Marcsisin, 2011).
3.D Summary

HIV-1 Vif is challenging to produce in quantities required for X-ray crystallography and NMR biophysical analysis. This fact has hampered advancement in the understanding of Vif biology. Recombinant Vif utilized in the structural studies presented in this Chapter was purified under denaturing conditions and renatured by dialysis. Vif produced by this procedure was assayed for biological functionality so that conformational data obtained using HX MS was relevant to a biological conformation of Vif. Vif has no intrinsic enzymatic activity and instead functions as an adapter protein that binds to other proteins and protein complexes. The work described in Section 3.B shows by multiple assays that recombinant Vif associated with the Src family kinases and altered kinase activity. The Vif:Hck interaction has been previously documented to be a biochemical function of Vif, therefore, the conformational information obtained with HX MS gives insight into that biochemically relevant conformation(s) in the context of Hck SH3 binding and kinase activation. The exact role of the Vif:Hck interaction for HIV replication and infection remains unclear, but it is interesting to think that the ability of Vif to alter kinase activity supports a meaningful role for Vif-induced regulation of Src-family kinases. One explanation for why Vif is such a potent activator of the SFKs is that there may be a connection between kinase activation and cell type restriction of APOBEC3 proteins. It was recently shown that phosphorylation of APOBEC3G by protein kinase A (PKA) decreased the intrinsic deaminase activity (Demorest, 2011) of APOBEC3G. Previous reposts have also shown that APOBEC3G can be phosphorylated in the presence of Fyn and Hck (Douaisi, 2005). Vif dependent SFK activation might be another mechanism by which HIV-1 counteracts the APOBEC3 proteins by regulating their phosphorylation state. Src-family tyrosine kinase
activation is an inherent biochemical property of recombinant Vif, which raises the possibility that compounds targeting the Vif:SFK interaction may exhibit antiviral activity.

The HX MS results for Vif are summarized in Figure 3.15A. To help visualize the deuteration into the different regions of Vif, the relative % unprotection from amide exchange after one minute in D₂O is shown for each peptic peptide of Vif. A low % unprotected value corresponds to a region that is not easily deuterated. Factors that could contribute to slowed exchange are secondary structural elements, quaternary structure, oligomeric complexes, and/or burying of amide hydrogens in the interior of the protein. The N-terminal portion of Vif which includes the binding sites for APOBEC3G/F and DNA/RNA displayed the least % unprotection. These results indicate that Vif contains structural elements which are primarily in the N-terminal region. Interestingly the N-terminal part of Vif that binds APOBEC3F/G displayed conformational heterogeneity in solution (striped bars and asterisks, Figure 3.15A), indicating the presence of multiple conformational states in solution. The conformational heterogeneity in this region and in the HCCH zinc finger might be a result of Vif adopting different conformational states required for binding different APOBEC3 proteins, nucleic acids, or other cellular targets. Another explanation for the conformational heterogeneity seen in Vif is that it may be the result of oligomerization in these particular regions.

A high percentage of unprotection indicates regions that are easily deuterated and solvent exposed and most likely do not contain stable higher order structural elements. The majority of the C-terminal portion of Vif became rapidly deuterated suggesting that the C-terminus is solvent exposed and unstructured in the absence of the E3 ligase machinery and zinc. The lack of structure in the absence of interacting partners in the C-terminus likely allows this region to
Figure 3.15 Implication from HX MS on Vif conformation. (A) Summary of deuterium incorporation into Vif after 1 minute in D$_2$O. Each bar represents a Vif peptic peptide. Shown is the relative % unprotected for each region of Vif. The residues that each peptide encompasses are indicated in the center of each bar and the maximum number of exchangeable amide hydrogens is indicated by the number in the top right of each bar. Peptides that displayed multiple populations in HX MS are indicated by the striped bar and asterisk (***) (B) Model of HIV-1 Vif based on the HX MS results. The N-terminal portion of Vif has structure while the C-terminal portion does not. The C-terminal portion of Vif becomes partially organized upon binding zinc and components of the E3 ligase machinery. Adapted from (Marciszin, 2011).
adopt different conformations in solution enabling Vif to associate with multiple binding partners such as Cullin 5, Elongin BC, Hck, and the APOBEC3 proteins.

The conformational results described in Section 3.C were used to create a cartoon model for Vif consistent with HX MS analysis as illustrated in Figure 3.15B. Vif likely contains an N-terminal folded core domain which acts as the adaptor region for APOBEC3F/G. The remainder of Vif is highly dynamic and mobile, allowing for recruitment of the E3 ligase machinery to facilitate poly-ubiquitylation of APOBEC3F/G, and for interaction with Src-family kinases. It is unknown whether these binding regions require structure to bind effectively to their targets or become structured upon binding.

The model in Figure 3.15A for Vif shows that the N-terminus was folded into a compact domain protected from deuteration, while the C-terminus was less structured and rapidly deuterated. Another explanation of why protection was observed in the N-terminus is that this region could be involved in oligomerization. Regardless, the model presented adds to the model presented by Auclair et al for full-length Vif as they determined that the N and C-termini of Vif are in close three dimensional proximity (Auclair, 2007). The only other structural models that exist for Vif are those presented by Lv et al and Balaji et al. Both of these models were constructed with homology modeling using protein templates with less than 25% sequence identity to Vif (Lv, 2007; Balaji, 2006). Shown in Figure 3.16A are the 10 second HX MS results overlaid onto the (i) Lv et al and (ii) Balaji et al models. Plotting HX MS results onto models allows for the analysis of the model validity. Regions protected from exchange should contain structure while regions that are rapidly deuterated should be unstructured and/or surface exposed. The images in Figure 3.16 show that for both models there are regions that agree with the HX MS results, and regions that do not. One region of significant difference between the two models
Figure 3.16 Vif model analysis. (A) Shown are the HX MS 10 second data for Vif mapped onto the (i) Lv et al and (ii) Balaji et al homology models (Lv, 2007; Balaji, 2006). The Vif SOCS box motif is highlighted in each model to illustrate differences. (B) Cartoon representation of the Vif HCCH zinc finger (i) and distance measurements between zinc finger residues for the (ii) Lv et al and (iii) Balaji et al homology models.
and HX MS results is the Vif SOCS box motif (highlighted in Figure 3.16A). HX MS analysis indicates that this region was unfolded in solution; however, both models indicate structural elements present. In the Lv et al model the SOCS box contains several alpha helices, while the Balaji et al model contains loops and beta sheets. The models are not consistent with each other or with HX MS results. One major empirical observation that the Lv et al and Balaji et al models overlooked is the ability of Vif to bind Zn$^{+2}$ through the HCCH zinc finger as illustrated in Figure 3.16B (i). Typically for proteins to efficiently coordinate zinc, the residues responsible for Zn$^{+2}$ coordination are within $\leq 3$ Å of the Zn$^{+2}$ ion (Alberts, 1998). A value of 3 Å would put the residues coordinating zinc in close proximity to each other. If distance measurements between the HCCH residues in Vif are made for the Lv et al and Balaji et al models (Figure 3.16B (ii and iii)), they indicate distances between the coordinating residues too large ($> 15$ Å) for efficient Zn$^{+2}$ coordination. The models shown in Figure 3.16 likely contain some structural features present in Vif; however, they do not accurately represent the structure of the full-length protein. Taken together, the results presented in the literature and the HX MS results described shed light on Vif conformation and suggest that Vif is a protein with two distinct regions: (1) the N-terminal APOBEC3 binding region that is likely structured and protected from amide exchange, and (2) the C-terminal region that is highly dynamic and solvent exposed which recruits the E3 ligase machinery.

3.E Materials and methods

3.E.1 SFK SH3 protein expression and purification

The Lck and Lyn SH3 domains were obtained from Dr. Thomas Wales (Northeastern University) which were produced as described in (Wales, 2006). For Hck SH3, the human gene
was over-expressed and purified as described previously (Poe, 2009). Human Hck SH3 W93A was sub-cloned from the psp72 Hck W93A YEEI plasmid and inserted into pET28b using the XhoI and NdeI restriction sites. Briefly, the *E. coli* strain Rosetta 2 DE3 pLysS was transformed with an SH3 expression plasmid and cells grown to OD<sub>600</sub> values of 0.6-1.0. Protein expression was induced by addition of 100 µM IPTG and allowed to proceed for 4 hours at 37 °C. Cells were harvested by centrifugation and lysed by sonication in 1X Phosphate buffered saline, pH 7.0. The lysate was centrifuged at 20,000 g for 40 minutes at 4 °C to remove any insoluble debris. GST-SH3 domains were then purified using GST affinity chromatography as described in Chapter 2. Hck SH3 W93A was over-expressed in *E. coli* Rosetta 2 DE3 pLysS cells as described above, and the soluble protein was purified with Ni-NTA affinity chromatography. The affinity tags were removed from the SH3 domains (wild type and W93A) by overnight cleavage with thrombin. All SH3 were dialyzed into Vif buffer (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.0) and stored at -80 °C. Protein concentrations were determined by Bradford assay (Bradford, 1976) and the purity verified with electrospray mass spectrometry (see Figure 3.5).

### 3.E.2 Z’-lyte SFK kinase activation assay

An overview of the Z’-lyte kinase assay can be found in Figure 3.8. All kinase assays were performed with the assistance of Dr. Lori Emert-Sedlak from the University of Pittsburgh. The Src family kinases Hck, Lck, and Lyn were expressed and purified in their down-regulated (by addition of the YEEI C-terminal tail) forms by the Smithgall laboratory (University of Pittsburgh) as described in (Trible, 2006). For the kinase assay, recombinant purified Vif (see Chapter 2 for Vif purification) was incubated with each SFK member at various molar ratios, and
kinase assays were performed using the FRET-based Z'-Lyte method and Tyr-2 peptide substrate according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Assays were performed in quadruplicate in 384-well plates in a final volume of 10 μL. Final ATP and Tyr-2 peptide substrate concentrations were held constant at 50 μM and 2 μM, respectively. Kinases were pre-incubated with Vif in kinase assay buffer for 30 min, followed by incubation with ATP and Tyr-2 peptide for 1 h and development buffer for an additional 1 h. Following reaction quench, substrate fluorescence was assessed on a Gemini XS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA).

3.E.3 Circular dichroism measurements

Circular dichroism measurements of denatured (Vif in 20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, 8 M guanidine HCl, pH 7.0) and renatured (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol pH 7.0) Vif were taken with a J-715 CD spectrometer (JASCO instruments, Easton, MD) with a path length of 1 mm at 20°C. Deconvolution of the CD spectra and assignment of the secondary structural percentages was conducted using the software program CDPro (Sreerama, 2000).

3.E.4 Intact HX analysis

Vif (30 μM) was thawed from -80 °C and incubated at 4 °C for 5 minutes before labeling. The Vif solution was diluted 15-fold with 20 mM MOPS, 150 mM NaCl, 1 mM DTT, 99.99% D₂O, pH 7.0 at 20 °C. For experiments that included zinc, Vif was equilibrated in the presence of 2 mM ZnCl₂ before labeling. The labeling reaction was quenched at various times by adjusting the pH to 2.6 with a 1:1 ratio of a solution containing 0.8 M guanidine HCl, 0.8%
formic acid, 100% H$_2$O. For SH3 binding experiments with recombinant Vif, wild-type Hck SH3 or W93A Hck SH3 were diluted to 6 μM and incubated with a 5-fold molar excess of Vif (30 μM) for 30 minutes at 4 °C. The mixture was labeled and quenched exactly the same as described for Vif alone. Samples were analyzed as described in Chapter 2.D and Appendix II.

3.E.5 Peptide HX analysis

Using a Shimadzu SCL-10A VP HPLC, protein samples were injected into an immobilized pepsin digestion column (2.1 mm x 50 mm stainless steel column packed with immobilized pepsin on POROS-20AL beads from PerSeptive Biosystems) flowing 0.05% formic acid (pH 2.6) at 20 °C at a flow rate of 200 μL/min. Peptic peptides produced in the pepsin column were trapped on a Michrom Bioresources (Auburn, CA) peptide micropeptide trap at 0 °C and desalted for 3 minutes before separation using a POROS 20 R2 (PerSeptive Biosystems) column. The column was 0.20 x 100 mm and was operated at 0 °C with a flow rate of 50 μL/min. A 12 minute 8-98% acetonitrile gradient (both mobile phases contained 0.05% formic acid, pH 2.6) was used to elute the peptides directly into a Waters QToF API US mass spectrometer with standard electrospray interface. Mass accuracy of <5 ppm was maintained through continuous lock mass correction and was carried out using Glu-fibrinogen peptide. Data were processed using the Excel-based software program HX-Express (as described in Chapter 2). Deuterium uptake for each peptide was determined by subtracting the centroid mass of each undeuterated control from the centroid mass of deuterium labeled samples. All peptide-level HX MS experiments were conducted in duplicate and the results averaged.
3F References


CHAPTER 4
Probing the Assembly of the HIV-1 Vif:Elongin BC Complex

Parts of this chapter have been published and are included in Appendix III:


4.A Introduction

4.A.1 Ubiquitylation and the Elongin BC-Cullin 5 ubiquitin ligase complex

HIV-1 Vif needs to assemble the Elongin BC dependent E3 ligase complex to fulfill its function of targeting APOBEC3G for ubiquitylation (Kobayahi, 2005; Mehle, 2004). Ubiquitylation is the process of covalently attaching the protein ubiquitin to a target protein. This can lead to several different fates of the ubiquitin tagged protein including endocytosis and degradation by the 26S proteasome (Pickart, 2001). The E3 ligase dependent ubiquitylation process is a three step mechanism which requires a ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and a ligase complex (E3) as illustrated in Figure 4.1A. The E1 activating enzyme forms a thiol ester bond with glycine 67 of ubiquitin which activates the C-terminal portion of ubiquitin for nucleophilic attack. The activated ubiquitin is transferred to the E2 conjugating enzyme and then to the target molecule through the formation of the E3 ligase complex [for a review on the ubiquitylation process see (Pickart, 2001)].

There are several types of E3 ligase complexes that are capable of protein ubiquitylation, however HIV-1 Vif utilizes the Elongin BC-Cullin 5 dependent E3 ligase complex for APOBEC3 ubiquitylation [for a review on E3 ubiquitin ligases see (Zimmerman, 2010)]. Figure 4.1B illustrates a cartoon depiction of the Vif E3 ligase complex. The Cullin 5 protein serves as
the main scaffolding component of the complex by bridging the target protein and its adaptor (APOBEC3G and Vif) to the ubiquitylation machinery (Rbx2 and the E2 enzyme). The function of the Elongin BC complex is to bridge Cullin 5 with the protein target (protein that is ubiquitylated) via association with the adaptor protein. For the Vif:APOBEC3G system, Vif acts as the adaptor protein for APOBEC3G (ubiquitylation target) by binding to the Elongin BC complex.

The human Elongin B and C proteins were originally identified not for their role in protein ubiquitylation, but as part of the heterotrimeric transcription factor Elongin ABC complex (Duan, 1995) that promotes transcription elongation by suppression of RNA polymerase II pausing (Conaway, 1995). Elongin B and C also have the ability to tightly bind with each other to create the heterodimeric Elongin BC complex. Several X-ray crystal and NMR structures have been solved for the Elongin BC complex bound to protein ligands. The structure of the Elongin BC complex (PDB:3DCG) is shown in Figure 4.1B and the protein ligand has been excluded to enhance visualization of the Elongin BC complex itself (Stanley, 2008). Elongin B is shown in green while Elongin C is shown in yellow. The region responsible for ligand binding is shown in gray and is localized to the C-terminal portion of Elongin C.

4.A.2 The Suppressor Of Cytokine Signaling box

The Elongin BC complex associates with adaptor proteins that contain a Suppressor Of Cytokine Signaling (SOCS) box (Bullock, 2006; Babon, 2008; Babon, 2009; Bullock, 2007). The SOCS box was first identified in the cellular SOCS family of proteins (SOCS1-7, CIS). The SOCS family is responsible for the modulation of intracellular responses from various cytokines by linking their specific cytokine related targets with the E3 ligase machinery for proteasomal
Figure 4.1 Ubiquitylation and the Elongin BC-E3 ligase complex.
(A) Ubiquitylation is a three step process involving an E1 activating enzyme (light blue), E2 conjugating enzyme (mustard) and an E3 ligase complex (highlighted by dashed box). Adapted from (Pickart, 2001). (B) A cartoon depiction of Elongin BC-E3 ligase complex is shown as well as a zoomed in view the Elongin BC heterodimer X-Ray structure (Stanley, 2008). Elongin B is shown in green and Elongin C in yellow. The ligand binding domain is also highlighted and the protein ligand for this structure has been excluded to enhance visualization of the Elongin BC complex.
targeting and subsequent degradation (Bullock, 2007). The linking of cellular SOCS box proteins (like Vif) to the E3 ligase complex is mediated through the Elongin BC complex. The canonical SOCS box motif contains three helices when bound to the Elongin BC complex and spans approximately thirty amino acids (Stanley, 2008). The sequence alignment of different SOCS box containing proteins (including Vif) is shown in Figure 4.2A. There are two separate regions of the SOCS box; the BC box which mediates binding to Elongin BC, and the Cullin box which determines the Cullin family member utilized for the core ligase scaffold (Stanley, 2008). The SOCS box motif binds tightly to the C-terminal portion of Elongin C mainly through hydrophobic interactions. As described above, there are numerous Elongin BC:ligand structures reported in the literature and shown in Figure 4.2B are the structures of VHL, Vif_{140-156}, and SOCS2 bound to the Elongin BC complex. The structures are shown for comparison with Vif and both VHL and SOCS2 show the three helical SOCS bundle, while the structure for Vif only contains the first helix encompassing the BC box which is responsible for Elongin BC interaction (Stanley, 2008). The Vif_{140-156}:Elongin BC structure is the only experimental three dimensional structure available for Vif to date (Stanley, 2008), which can likely be attributed to the unstructured dynamic nature of Vif as described in Chapter 3. The Vif SOCS box was not completely resolved in this structure as only electron density for residues encompassing the BC box was observed. The importance of the formation of the Vif:Elongin BC complex for the degradation of APOBEC3G makes understanding the structural consequences of this interaction extremely important. The subsequent sections of Chapter 4 describes the use of HX MS, CD, and pull-down analysis to biophysically and biochemically characterize the Vif:Elongin BC interaction.
Figure 4.2 Suppressor of Cytokine Signaling box motif. (A) Sequence alignment of SOCS box containing proteins and structure of the VHL SOCS box (Hon, 2002; Stanley, 2008). Conserved and similar residues are shown in red. Cellular SOCS box proteins contain a conserved cystine (red) residue in helix-1 which is replaced by alanine (green) in Vif. (B) Structures of the VHL, Vif_{140-156}, and SOCS2 Elongin BC complexes. (Hon, 2002; Stanley, 2008; Bullock, 2006).
4.B *E. coli* production of the Elongin BC complex and Vif variants

Prior to characterization of the Vif:Elongin BC complex, it was necessary to express and purify the Elongin BC complex before biophysical analysis could occur. One successful strategy for expression of the Elongin BC complex was developed by the Knapp laboratory at the University of Oxford and is illustrated in Figure 4.3A (i). The genes for Elongin B and C were cloned into one *E. coli* expression plasmid and the resulting expression vector transformed into *E. coli*. The work done by Bullock *et al* utilized the Elongin BC vector to co-express Elongin BC along with its binding partner SOCS2 (Bullock, 2006). To conduct conformational studies of the Elongin BC with Vif, the Elongin BC complex needed to be expressed and purified without Vif, SOCS, or any other binding proteins. This was done by obtaining the Elongin BC plasmid from the Knapp laboratory and transforming it into *E. coli*. Protein expression was conducted for 20 hours at 18 °C. Expression was monitored with SDS-PAGE as shown in Figure 4.3A (ii). Bands corresponding to the molecular weights of Elongin B and C (13 kDa and 10 kDa respectively) appeared over the course of the induction experiment suggesting Elongin B and C were co-expressed.

The Knapp laboratory was able to purify the Elongin BC bound to SOCS2 because SOCS2 contained a 6X histidine affinity tag. The SOCS2:Elongin BC complex was purified using Ni-NTA affinity purification, as 6X histidine has high affinity for the Ni$^{2+}$ ion (see Chapter 2.7 for Ni-NTA affinity purification overview). The Elongin BC utilized in these studies was not bound to any other protein and did not contain an affinity purification tag. A two step purification process of anion exchange and size exclusion chromatography was used to purify the Elongin BC complex. Purification results are shown in Figure 4.3B and C. The chromatograms for each step are shown in Figure 4.3B and C (i) and SDS-PAGE analysis of collected fractions...
Figure 4.3 Expression and Purification of recombinant Elongin BC complex. (A) (i) pACYCDUET-ELBC vector used for co-expression of Elongin BC (Bullock, 2006). (ii) Elongin BC induction gel. (B) (i) Chromatogram from the anion exchange purification step to isolate the Elongin BC complex. Fractions from (i) were analyzed with SDS-PAGE and fractions containing Elongin BC (ii) pooled. (C) (i) Pooled Elongin BC was purified further using size exclusion chromatography purification (i) and monitored by SDS-PAGE(ii). Adapted from (Marcisisin, 2010).
in Figure 4.3B and C (ii). The results in Figure 4.3 show that Elongin B and C were successfully isolated and remained bound to each other through the multi-step purification process. The fractions containing purified Elongin BC complex shown in Figure 4.3C (ii) were pooled and subjected to ESI-MS analysis for mass verification as shown in Figure 4.4A.

A series of Vif variants were also produced for this study in order to fully characterize the interaction of Vif and the Elongin BC complex. Several variants of Vif that contained mutations in the region necessary for Elongin BC binding were produced using site directed mutagenesis [see (Weiner, 1994) for overview of mutagenesis]. The variants Vif_{145A} and Vif_{1-141} were chosen because mutation of the conserved leucine residue in the Vif SOCS box (residue 145) has been shown to severely reduce Elongin BC:Vif binding (Mehle, 2004; Stanley, 2008) and Vif_{1-141} does not contain the SOCS box. Vif_{1-141} was chosen to serve as a negative control for Elongin BC binding. Both of these mutants were expressed and purified using the same procedure utilized for wt full-length Vif as described in Chapter 2. In addition to probing the binding between the recombinant Vif variants and the Elongin BC complex, a peptide encompassing the the Vif BC box (Vif_{135-158}) was purchased. All Vif variants were subjected to ESI-MS analysis for mass verification as shown in Figure 4.4B-E. A cartoon depiction of the Vif variants utilized is shown in Figure 4.5A.

4.C Characterization of HIV-1 Vif:Elongin BC binding

4.C.1 Pull-down analysis

After producing the Elongin BC complex and different Vif variants, the Vif:Elongin BC interaction was tested using pull-down experiments [for an overview on pull-down assays see (Vikis, 2004)]. For Vif:Elongin BC pull down experiments, each Vif variant was immobilized
Figure 4.4 ESI-MS analysis of Elongin BC and Vif variants. Shown are the intact mass spectra (i) and deconvoluted spectra (ii) for purified Elongin BC (A), wt full-length Vif (B), and different Vif SOCS box variants (C-E). (Marcisisin, 2011)
onto Ni-NTA agarose and the Vif loaded agarose incubated with recombinant Elongin BC. After a 30 minute incubation period, unbound Elongin BC was washed away and the reaction mixture analyzed using SDS-PAGE as shown in Figure 4.5B. The gel in panel B (i) shows the different Vif variants experimental inputs and the gel in (ii) shows representative data from one set of pull-down experiments. The gel in (ii) definitively shows that wt full-length Vif and Vif\textsubscript{135-158} associated with Elongin BC by the presence of Elongin B and C bands. The lack of Elongin C bands in the lanes corresponding to Vif\textsubscript{L145A} and Vif\textsubscript{1-141} indicates that these variants had decreased affinity for the Elongin BC complex. Elongin B could not be used for comparison because Vif\textsubscript{1-141} ran at the same apparent molecular weight as Elongin B as indicated by the red asterisks. To further quantify the Vif:Elongin BC binding observed in pull-down gels, densitometry measurements were made and binding assessed using the Elongin C bands from the gel in Figure 4.5B (ii). The relative Elongin C band intensities from all pull-down experiments (indicative of Vif:Elongin BC binding) were normalized to the Elongin C band intensity in the presence of wt Vif. Results from densitometry measurements are shown in Figure 4.5C and indicate that removal of the SOCS box severely impaired Elongin BC binding. HIV-1 viruses containing the Vif\textsubscript{L145A} variant have been shown by viral infectivity assays to have reduced viral infectivity (Yu, 2004; Mehle, 2004) and in these \textit{in vitro} pull-down assays there was a three fold reduction in Elongin BC binding to Vif\textsubscript{L145A} compared to wt Vif. Previous studies have suggested that mutation of leucine 145 to alanine abolished Vif:Elongin BC binding (Stanley, 2008; Yu, 2004). The residual binding observed in these pull-down experiments is likely explained by the contacts made with Elongin C by the remainder of the Vif SOCS box. In other SOCS proteins (e.g., SOCS-2 and 4) the region C-terminal to the conserved leucine (Vif L145) forms two additional helices (see Figure 4.2) that interact with Elongin C (Bullock, 2007;
Figure 4.5 Pull-down analysis of Elongin BC and Vif variants. (A) Vif variants used for testing the interaction with the Elongin BC complex (not to scale). (B) SDS-PAGE gels of 1 nmol (i) recombinant Vif constructs and the (ii) pull down analysis between Vif variants and the Elongin BC complex (3 nmols of Elongin BC were used) Binding was assessed using the Elongin C band due to interference between Elongin B and Vif1-141 bands running at the same apparent molecular weight (indicated by the *). (C) Densitometry measurements of the Elongin C band in panel B (ii). Error bars were determined using two replicate analyses. (Marcasin, 2010)
Bullock, 2006). The Vif\textsubscript{140-156} crystal structure only contained electron density for residues of the Vif BC box. The remaining residues (not resolved in the crystal structure) of the Vif SOCS may interact with the Elongin BC complex via contacts not apparent in the crystal structure and could account for the partial binding observed for Vif\textsubscript{145A}. Interestingly, the Vif\textsubscript{135-158} peptide had the most binding to the Elongin BC complex in these pull-down experiments indicating Vif\textsubscript{135-158} had higher affinity for the Elongin BC complex as compared to wt full-length Vif. The work presented in this section demonstrates that recombinant Vif and Elongin BC are functionally active and able to interact with one another in vitro.

### 4.C.2 Intact HX MS analysis

The conformational consequences of full-length recombinant Vif interacting with the Elongin BC complex were assayed with HX MS. The conformational changes in Elongin B and C as a result of Vif binding were first assayed by incubating Elongin BC with a four-fold molar excess of full-length Vif and monitoring deuteration. Intact HX MS results for Elongin B and C in the absence and presence of Vif are shown in Figure 4.6. The +9 charge state of Elongin C is shown as it became deuterated with (A) or without (B) Vif. Upon Vif incubation, the mass increase of Elongin C was suppressed as a result of the Vif:Elongin BC complexation. Figure 4.6C shows the deuterium uptake curves for Elongin C which indicate that 18 residues were protected from deuteration upon complexation with Vif. Several factors could contribute to such protection including stabilization of the Elongin C ligand binding domain or the protection of backbone amide hydrogens by the presence of Vif. In addition to monitoring Elongin C, the Elongin B conformation was also probed. Deuteration into Elongin B (Figure 4.6D) was not as dramatically altered by Vif as was in the case for Elongin C, but Elongin B did show a slight
Figure 4.6 Intact HX MS Elongin BC results. (A) Mass spectra of the +9 charge state of Elongin C in the absence of Vif, or (B) incubated with a four fold molar excess of Vif. The dotted lines are provided to guide the eye and are fixed at 1222 m/z in both panels. (C) Relative deuterium uptake curves for Elongin C and (D) Elongin B. The solid line represents the unbound state and the dotted line is in the presence of HIV-1 Vif. The error of intact HX MS measurements was ± 2 Da. (Marcsisin, 2010)
decrease in deuteration upon Vif complexation. The decrease in Elongin B deuteration could result from conformational changes in Elongin C, perhaps as a result of stabilization of the Elongin BC heterodimeric interface. An alternative explanation is that other than the Vif:Elongin C binding interface, a portion(s) of Elongin B physically interacted with Vif. Taken together, these results indicate that upon incubation with full length recombinant HIV-1 wt-Vif in vitro, the conformation of both Elongins in the Elongin BC complex were altered.

To determine if Elongin BC binding resulted in any major conformational changes in Vif, the intact HX MS experiments described above were altered and conducted by incubating Vif with a four-fold molar excess of Elongin BC. Intact HX MS results for Vif are shown in Figure 4.7. The +26 charge state is shown for Vif for each deuteration time in the absence (A) or presence (B) of the Elongin BC complex. The deuteration profiles of Vif are similar in both instances (unbound and bound) and the deuterium incorporation curves (C) indicate that no detectable conformational changes in full-length Vif occurred upon Elongin BC binding. Along with the global HX measurements made for Vif in the presence of excess of Elongin BC, pepsin digestion was also utilized to investigate if any conformational changes could be observed in the regions responsible for Elongin BC binding. These experiments were inconclusive because there was incomplete peptide coverage for the region in Vif responsible for interaction with Elongin BC complex upon complex formation.

To further investigate and determine if Elongin BC binding could alter the conformation of the Vif BC box region, a synthetic Vif peptide was utilized that encompassed the region necessary for Elongin BC binding (Vif135-158). Vif135-158 was subjected to HX MS analysis and results are shown in Figure 4.8 in the absence (A) or presence (B) of a four fold molar excess of Elongin BC. The mass migration for the Vif135-158 peptide alone to the higher deuteration point
Figure 4.7 Intact HX MS Vif results. (A) Mass spectra of the +26 charge state of Vif, or (B) incubated with a four fold molar excess of Elongin BC. The dotted lines are provide to guide the eye and are fixed at 870 m/z. (C) Relative deuterium uptake curves for Vif. The solid red line represents the bound state and the dotted black line is in the absence of Elongin BC. The error of intact HX MS measurements was ± 2 Da. Adapted from (Marcsisin, 2010).
occurred after only 10 seconds in D$_2$O. Deuteration for the Vif$_{135-158}$ peptide was suppressed in the presence of Elongin BC and reached the deuteration level of the unbound form after 20 minutes in D$_2$O. The deuterium incorporation curves are shown in Figure 4.8C. The data for the unbound form of the peptide indicate that Vif$_{135-158}$ did not contain any structural elements in its unbound state. The red curve in Figure 4.8C corresponds to the bound form of Vif$_{135-158}$ and differs from the unbound form by 10 Da at the 10 second time point. The reduction in deuteration between bound (red) and unbound (black) Vif$_{135-158}$ is consistent with changes in solvent accessibility and hydrogen bonding, indicative of formation of structure in the presence of the Elongin BC complex.

While intact HX MS analysis of full length Vif did not indicate if significant global conformational changes occurred upon Elongin BC binding (Figure 4.7), the Vif BC box (Vif$_{135-157}$) did appear to change upon Elongin BC incubation (Figure 4.8). A plausible explanation as to why changes (decreased deuterium incorporation) are seen in Vif$_{135-158}$ but not in full length Vif could be that other conformational changes occurred in the full length protein that lead to increases in deuterium incorporation. Despite the lack of changes in full length Vif, the reduction in deuteration for Vif$_{135-158}$ indicates that structural elements formed in Vif$_{135-158}$ upon Elongin BC binding. Interestingly, other folded SOCS box motifs have been observed in structures with the Elongin BC complex (Babon, 2008; Bullock 2007) and it was reported that the SOCS box of SOCS3 folded into the helical structure observed with NMR upon complexation with Elongin BC (Babon, 2008). The HX MS results for Vif$_{135-157}$ are consistent with the known hypotheses that SOCS box proteins need to fold to bind Elongin BC and/or fold upon binding.
Figure 4.8 Intact HX MS Vif135-158 results. Deuteration of Vif$_{135-158}$ in (A) the absence of the Elongin BC complex or (B) the presence of the Elongin BC complex. The $+5$ charge state of Vif135-158 is shown. (C) Deuterium uptake curves for data shown in panels A and B. The error of intact HX MS measurements was $\pm 2$ Da. Adapted from (Marcisin, 2010).
4.C.3 CD analysis of Vif\textsubscript{135-157}

The deuteration of unbound Vif\textsubscript{135-157} in section 4.C.3 suggests that the Vif BC box was unstructured in the absence of Elongin BC. Circular dichroism measurements were made to further probe the secondary structural elements of the Vif\textsubscript{135-157} synthetic peptide in the unbound state. The results of the CD measurements are shown in Figure 4.9A. The red CD trace represents Vif\textsubscript{135-157} in phosphate buffer without Elongin BC. The Vif\textsubscript{135-157} trace shows a minimum below 200 nm indicative of irregular secondary structure (Kelly, 2005) which is consistent with HX MS results of unbound Vif\textsubscript{135-157}. The Vif\textsubscript{135-157} peptide was then incubated with increasing concentrations of the known helix inducing agent 2,2,2-trifluoroethanol (TFE) (Sonnichen, 1992). TFE as a co-solvent is thought to induce helix formation by forming a TFE solvent cluster around the peptide excluding water and solvent hydrogen bonding. This exclusion of solvent hydrogen bonding promotes peptide intramolecular hydrogen bonding and secondary structure formation (Roccatano, 2002). The CD spectra for Vif\textsubscript{135-157} in 5% TFE (dotted line), 20% TFE (dashed line), and 80% TFE (blue line) are shown in Figure 4.9A. The addition of increasing concentrations of TFE shifted the Vif\textsubscript{135-157} single minimum from 190 nm (0% TFE) to two minima at 205 and 222 nm respectively (80% TFE). The change in minima and shape of the CD spectra in the presence of TFE indicate that Vif\textsubscript{135-157} was unstructured in the absence of TFE and TFE induced structure.

To further quantify the changes in secondary structure of Vif\textsubscript{135-157}, the software program K2D2 (Iratxeta, 2008) was used to estimate the relative percentage of secondary structure using the CD results in Figure 4.9A. The K2D2 outputs are shown in Figure 4.9B and indicate the percentages of alpha helix, irregular structure, and beta sheet secondary structural elements. The results in Figure 4.9 show that at low TFE concentrations, Vif\textsubscript{135-157} contained mostly irregular
Figure 4.9 CD analysis of Vif₁₃₅₋₁₅₇. (A) The CD spectra for Vif₁₃₅₋₁₅₇ in phosphate buffer (red), 5% TFE (dotted), 20% TFE (dashed), and 80% TFE (blue) are shown. Note the 5% spectrum is not shown. (B) Secondary structural analysis of Vif₁₃₅₋₁₅₇ in increasing concentrations of TFE. The percentage of alpha helix (green), irregular structure (copper), and beta sheet (black) present at the different TFE concentrations are shown.
secondary structure. The percentage of alpha helix content increased with higher TFE concentrations. The CD measurements described above for Vif_{135-157} are consistent with HX MS results described in Section 4.C.2 and indicate that the Vif BC box was unstructured in solution.

4.C.4 HX MS titrations

The work presented in 4.C.1 and 2 established that recombinant Vif interacted specifically with Elongin BC resulting in conformational changes that were detected by HX MS in Elongin BC and the Vif BC box. HX MS was then utilized to determine the dissociation constants ($K_d$) between the Elongin BC complex and the different Vif variants. The affinity of full length HIV-1 Vif with the components of the E3 ubiquitin ligase has not been previously reported in the literature. Protein conformation monitored by HX MS can be used as a probe of $K_d$ values for protein-ligand interactions that are not easily assayed by other biophysical techniques (Engen, 2003). The process of protein:ligand $K_d$ approximation using HX MS is illustrated in Figure 4.10. The assay begins with incubation of a fixed amount of Elongin BC with increasing amounts of each Vif variant. The protein mixtures were then pulsed-labeled [see (Deng, 1999; Wales, 2006) for review] for 10 seconds in D$_2$O, quenched, and mass analyzed. HX MS titrations require an observable mass shift (conformational change) in the protein that is in fixed quantity. Elongin C conformation was monitored and used as a result of the large conformational change that is observed between free and Vif bound forms. Two separate mass distributions were observed. The shift in populations between free and Vif bound forms of Elongin C was due to the varying amounts of each form present in solution as a result of increasing Vif concentrations. The percentages of free vs. Vif bound Elongin C were calculated and titration curves constructed. Mass spectra for the HX MS titrations are shown in Figure 4.11.
**Figure 4.10 HX MS titration assay.** Varying amounts of Vif were added to a fixed concentration of Elongin BC and protein mixtures were pulse-labeled for 10 seconds then quenched. Samples were mass analyzed and binding curves for Vif:Elongin BC constructed. Adapted from (Marcsisin, 2010).
Figure 4.11 Vif:Elongin BC HX MS titration mass spectra. The +7 charge state of Elongin C is shown in the presence of each Vif variant at concentrations indicated. The blue distribution represents the unbound population of Elongin C and the red distribution represents the bound population. The error of intact HX MS measurements was ± 2 Da. Adapted from (Marciszin, 2010).
The spectra for the wt Vif:Elongin BC interaction (Figure 4.11A) indicate that with increasing amounts of Vif, the amount of unbound Elongin C decreased and the amount of bound Elongin C increased. The bound form was heavily protected from deuteration (red dotted line), while the unbound form was not as protected (blue dotted line). The bound form of Elongin C was the only form observed at wt Vif concentrations above 3 µM. The spectra for (B) VifL145A, (C) Vif135-158, and (D) Vif1-141 are also provided in Figure 4.11. These data show similar Elongin C deuteration patterns; albeit, at different concentrations of each Vif variant incubated with Elongin BC as compared to experiments using wt Vif. The percentage of bound Elongin C was graphed vs. Vif concentration (µM) and the graphs fitted with a sigmoidal function as illustrated in Figure 4.12. By using the graph in Figure 4.12 where 50% of Elongin C was bound, the dissociation constants could be calculated as illustrated for wt Vif. The dissociation constant for the wt Vif:Elongin BC interaction was calculated to be 1.9 ±0.2 µM from replicate titration experiments. The HX MS derived dissociation constant is in agreement with the work of Bergeron et al of 1.19 µM for the Vif variant Vif130-180 measured with ITC (Bergeron, 2010).

Previous studies suggest that Vif L145A abolishes the interaction between Vif and the Elongin BC complex (Yu, 2004; Stanley, 2008). The results shown in Figure 4.11 and Figure 4.12 indicate that VifL145A still interacted with the Elongin BC complex in vitro, however, had decreased affinity for the Elongin BC complex as compared to wt Vif. The HX MS derived dissociation constant for VifL145A was 3.9 µM ±0.1 µM and is in agreement with 4.6 µM recently reported for the Vif variant Vif139-192 L145A (Wolfe, 2010). There was no binding detected between Vif1-141 and the Elongin BC complex using the HX MS titration assay. This negative binding result was expected as Vif1-141 lacks the Viral BC box required for interaction with Elongin BC. Vif135-158 showed the highest affinity for the Elongin BC complex with a dissociation constant of 0.23 µM.
Figure 4.12 Vif:Elongin BC HX MS titration curves. The titration curves for Elongin BC and each Vif variant are shown and the calculated dissociation constant provided. The errors reported for each $K_d$ were determined from duplicate $K_d$ determinations. Adapted from (Marcisin, 2010).
±0.04 μM, which is nearly a 10-fold increase in affinity for the Elongin BC complex as compared to wt Vif. This increase in affinity of Vif\textsubscript{135-158} for Elongin BC as compared to wt Vif might be explained by steric effects that arise from the extra residues present in full-length protein as compared to the Vif\textsubscript{135-158} peptide. The results for Vif\textsubscript{135-158} are consistent with the pull-down results in Section 4.C.1 and indicate that the Vif BC box is able to specifically bind to the Elongin BC complex \textit{in vitro}. These results for the Vif:Elongin BC \textit{in vitro} interaction presented in this chapter are in stark contrast to other cellular SOCS that do not interact with Elongin BC \textit{in vitro}. Babon \textit{et al} showed that SOCS1-7 required co-expression with Elongin BC in order to form the cellular SOCS:Elongin BC complex (Babon, 2009). The ability of Vif to bind Elongin BC \textit{in vitro} might be a unique property that allows Vif to escape an additional regulatory mechanism that prevents cellular SOCS proteins from binding with Elongin BC without the aid of other cellular components. This property of Vif might ensure that HIV-1 has the ability to hijack the E3 ligase machinery and maintain effective viral replication.

4.C.5 Peptide HX MS analysis of the Elongin BC Complex

The work presented in Section 4.C.2 characterized the Vif:Elongin BC interaction on the intact protein level. Pepsin digestion post deuterium labeling was utilized to further probe the Elongin BC complex. Peptide analysis allowed for the determination of which regions in Elongin B and Elongin C were dynamic and determined how these regions may change conformation in the presence of Vif.

The peptides produced with pepsin digestion and utilized for deuteration measurements are listed in Table 4.1. The deuterium uptake curves for the peptides in Table 4.1 are shown in Figure 4.13. The deuterium uptake curves in Figure 4.13 show the deuteration for the different
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<th>End</th>
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<th>Peptide Sequence</th>
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**Table 4.1 Peptic peptides of the Elongin BC complex.** Peptides above were utilized for deuterium incorporation measurements.
Figure 4.13 Elongin BC peptide deuterium incorporation curves. All peptides that could be followed for deuteration measurements are shown. Some peptides do not have data in the presence of Vif due to interference from Vif peptic peptides. The error of peptide HX MS measurements was ± 0.25 Da. Adapted from (Marcisin, 2011).
regions in Elongin B and C in the Vif unbound and bound state. This section will focus on the Elongin BC results for the Vif unbound state. The HX MS results for Elongin BC are summarized in Figure 4.14. The HX MS results for Elongin BC are summarized in deuteration maps as was done for Vif in Chapter 3. Shown is the primary structure for each Elongin and the corresponding secondary structural elements as determined from the X-ray structure PDB:3DCG (Stanley, 2008). Below the primary structure are bars that represent each of the Elongin B and C peptic peptides. The bars are color coded to their relative % deuteration (see Chapter 3, Equation 3.2 for calculation). The regions where Elongin C makes contact with Elongin B in the formation of the Elongin BC heterodimer showed the lowest deuteration. These regions in Elongin C included the first beta sheet (S1), loop one (L1) and a section of helix three (H3) which were each $\leq 30\%$ deuterated after 20 minutes in D$_2$O. Residues 29-62 in the N-terminal region of Elongin C, including S2, S3, H1, H2, L2 and L3, were $\geq 50\%$ deuterated after 20 minutes in D$_2$O. The C-terminal ligand binding domain of Elongin C, including a section of H3, L5 and H4, was also heavily deuterated ($\geq 60\%$ deuterated after 20 minutes).

In contrast to Elongin C, most of Elongin B was protected from deuteration. Residues 1-85 of Elongin B are part of the N-terminal ubiquitin-like domain of Elongin B, while residues 86-118 constitute the C-terminal tail (Brower, 1999). As shown in Figure 4.14A, residues 1-62 were $\leq 40\%$ deuterated after 20 minutes in D$_2$O. The C-terminal portion of Elongin B was much more readily deuterated. Residues 88-116 became $\geq 60\%$ deuterated after 20 minutes in D$_2$O. These results suggest that the C-terminal portion of Elongin B was dynamic, solvent exposed and not hydrogen bonded in solution. There is a lack of structural data on the C-terminal portion of Elongin B and the peptid HX MS results for the Elongin B C-terminal tail explain why there is
Figure 4.14 HX MS peptic peptide summary for Elongin BC. (A) Representative peptides spanning the length of the Elongin C and Elongin B proteins are shown as color-coded bars below the primary structure. The secondary structural elements are indicated by: boxes for helices (H), arrows for beta sheets (S) and lines for loops (L). (B-E) Mass spectra for selected peptides. The error of peptide HX MS measurements was ± 0.25 Da. Adapted from (Marcosin, 2010)
a lack of data as such a dynamic region would be difficult to observe with crystallography and NMR. The deuteration for Elongin B selected peptides is shown in Figure 4.14D and E.

The one minute HX MS peptide results for Elongin B and C were overlaid onto the Elongin BC structure as shown in Figure 4.15. It is important to remember that the structures for Elongin BC complex have only been solved when bound to a SOCS ligand. The HX MS results presented in this section are the first data of the Elongin BC complex in the unbound form. HX MS results in Figure 4.15 demonstrate that Elongin B was protected from deuteration and likely adopted a conformation similar to that observed in the bound X-ray structure. Elongin C is quite different in that it became rapidly deuterated in the unbound state. The conformation of Elongin C in the unbound state is likely dynamic and unstable [as previously reported for Elongin C in the absence of Elongin B and SOCS ligands (Botuyan, 1999; Botuyan 2001)] and explains why there are no structures of the Elongin BC complex in the absence of SOCS ligands.

4.C.6 Localization of conformational changes in Elongin BC

Having probed the conformation of the Elongin BC complex alone using HX MS, the conformation of the Elongin BC in the presence of Vif was studied next. To determine where Vif stabilized the Elongin BC complex upon binding, Elongin BC was mixed with a four-fold molar excess of (as described above for intact analyses) Vif and labeled with deuterium. Deuteration results for Elongin BC incubated with Vif are summarized in Figure 4.16. The deuteration for the peptide corresponding to the Elongin C SOCS ligand binding region (residues 76-100) is shown in Figure 4.16A. The deuteration of this peptide in the absence and presence of Vif is markedly different. The mass distribution in the absence of Vif shifted to the right of the dotted line (fixed at 929 m/z, provided as a visual reference) after only one minute in D₂O, while the mass
Figure 4.15 HX MS inferences of the unbound Elongin BC structure. One minute HX MS data mapped onto the Elongin BC structure. The colored dotted line indicates residues un-resolved in the X-ray structure for which there was deuterium incorporation data. The dashed line indicates the boundary between Elongin C and Elongin B. Adapted from (Marcisisin, 2010).
Figure 4.16 Deuterium incorporation changes in Elongin BC. (A) Deuteration of the Elongin C peptide encompassing residues 76-100 in the absence and presence of full length Vif. (B) Deuterium uptake curves for peptides in Elongin B and C displaying changes upon Vif incubation. (C) and (D) Location of conformational changes in Elongin B and C upon Vif incubation mapped onto the Vif140-156 X-ray structure (Stanley, 2008). A cartoon depiction of the uncharacterized regions of Vif is shown in the surface representation. The error of peptide HX MS measurements was ± 0.25 Da. Adapted from (Marciszin, 2010).
distribution of the same peptide in the presence of Vif never shifted to the right of the dotted line over the timescale of these measurements. Deuterium uptake curves for this peptide and other Elongin BC peptides displaying changes in deuterium upon incubation with Vif are shown in Figure 4.16B. Several regions in Elongin C had large decreases in deuteration (2-6 Da) upon Vif complexation. These regions included residues 29-46 (S2, H1, L2, H2), 76-100 (H3, peptide shown in Figure 4.16A L5) and 105-109 (H4) which lie within the Elongin C SOCS binding pocket. The changes in deuteration for Elongin B and C are highlighted on the cartoon (Figure 4.16C) and surface representation (Figure 4.16D) of the Vif\textsubscript{140-156}:Elongin BC X-ray structure (Stanley, 2008). The results show that the majority of conformational changes in Elongin B and C localize to the Vif binding interface.

Unlike Elongin C, Elongin B displayed more subtle changes in deuteration. The only region in Elongin B that displayed deuterium incorporation changes upon Vif incubation was residues 63-79 (S5). The changes in deuteration for Elongin B residues 63-79 are indicative of changes in protein dynamics as this region started out at similar deuteration levels which diverged over time (Morgan, 2009). The decreased dynamics of this region of Elongin B upon ligand binding can likely be explained by its proximity to the ligand binding region of Elongin C (H3, L5, and H4) which becomes stabilized by Vif binding.

4.D Summary

Previous studies provide evidence that the HIV-1 Vif:Elongin BC interaction occurs \textit{in vivo} and is essential for the degradation of APOBEC3 enzymes (Yu, 2004; Kobayashi, 2005). The results presented in this Chapter show that full length recombinant renatured Vif specifically bound the Elongin BC complex \textit{in vitro} and did not require other cellular components. A
cartoon depicting the Vif:Elongin BC interaction as revealed by HX MS is shown in Figure 4.17. Elongin C is dynamic in the absence of SOCS ligands while Elongin B is conformationally stable. The dissociation constant of wt Vif for the Elongin BC complex was measured by HX MS to be 1.9 µM. Vif binding stabilized Elongin C and a small region of Elongin B that makes contact on the backside of the Elongin C ligand binding region. The peptide encompassing the Vif BC box folds upon binding. Vif seems to be the first SOCS box containing protein that has the ability to associate with the Elongin BC complex without the aid of other cellular factors or co-expression in vivo. Numerous cellular SOCS proteins such as the VHL tumor suppressor and SOCS 1-7 only bind to the Elongin BC complex when co-expressed in vivo (Babon, 2008; Feldman, 1999). The interaction of cellular SOCS proteins with the Elongin BC complex seems to be mediated through other cellular components not present during in vitro experiments as the formation of the VHL:Elongin BC complex requires the chaperonin TRiC. The ability of Vif to specifically interact with the Elongin BC complex independent of other cellular factors might be a unique property of Vif that allows it to more easily hijack the E3 ligase machinery.

Global conformational changes in full-length Vif upon incubation with a four-fold molar excess of Elongin BC were not detected; however, deuteration into the Vif_{135-158} peptide changed dramatically upon Elongin BC incubation suggesting that the Vif BC box folded upon binding and likely does so in context of the full length protein. Such an event would be consistent with cellular SOCS proteins such as that observed for SOCS3 (Babon, 2008). The ability of Vif to specifically interact with the Elongin BC complex independent of other cellular factors is a unique and promising target for anti-HIV-1 drug design. The ability of HX MS to detect Vif:Elongin BC binding provides a unique tool to screen for Vif:Elongin BC inhibitors (described in Chapter 5).
Figure 4.17 Model for Elongin BC:Vif interaction. Cartoon of Vif:Elongin BC interaction as revealed by HX MS. (Marcisin, 2010)
4.E Materials and methods

4.E.1 Protein expression and purification

Full length HIV-1 HXB2 Vif, Vif L145A, and Vif1-141 were over-expressed and purified using the pET28b vector as described in Chapter 2. The Vif L145A, and Vif1-141 variants were constructed using Lightning QuickChange® site directed mutagenesis from the wt HIV-1 Vif template. Human Elongin B and C (residues 17-122) were co-expressed using the pACYCDUET-ElCB vector in BL21 (DE3) cells. The pACYCDUET-ElCB vector was a kind gift from Dr. Stefan Knapp at the Structural Genomics Consortium (University of Oxford). Starter cultures (50 mL) of BL21 (DE3) cells containing the pACYCDUET-ElCB vector were grown overnight at 37 °C in the presence of the appropriate antibiotics. The starter cultures were then used to inoculate 500 mL of LB (with antibiotics) in 1 L flasks and grown for approximately 1-2 hours at 37 °C until OD600 values of ~ 1 were reached. Protein expression was then initiated by addition of 50 μL of 1 M IPTG (final diluted concentration of 0.1 mM) and allowed to proceed overnight at 18 °C. Induction samples were collected throughout the experiment and analyzed by SDS-PAGE, Cells were then harvested by centrifugation and stored at -80 °C until purification. Elongin BC containing cell pellets were then re-suspended in 10 mL of anion exchange buffer A (see below) supplemented with PMSF and lysed with sonication. Lysozyme was then added to the cells and incubated for 30 minutes on ice to ensure complete lysis. The soluble protein fraction was isolated by centrifugation at 30, 000 g for 30 minutes. Soluble Elongin BC was purified by anion-exchange on a HiTrap QHP column (FPLC flow rate of 0.25 mL/min, 0-1M NaCl gradient over 15 column volumes) using the following buffers: Buffer A (50 mM Tris HCl, 1 mM MgCl2, pH 8.0) and Buffer B (50 mM Tris HCl, 1 M NaCl, 1 mM MgCl2, pH 8.0). Fractions were analyzed by SDS-PAGE and Elongin BC containing
fractions pooled and concentrated to ~ 500 μL using an YM3 Centricon spin column (Millipore, Billerica, MA). Elongin BC was further purified by size exclusion chromatography on a Superose 12 10/300 column (GE Healthcare) (FPLC flow rate of 0.25 mL/min) with the following buffer: (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.0). Fractions were analyzed by SDS-PAGE and Elongin BC containing fractions pooled; ESI mass analyzed, and stored at – 80 °C.

The Vif 135-158 peptide (HHHHHHPQAGHNKVGSLQYLALAALITPKK) was designed from the HXB2 Vif viral BC box sequence and purchased from Gen Script (Piscataway, NJ). The peptide (>98% purity) was dissolved directly into Vif buffer (20 mM MOPS, 150 mM NaCl, 1 mM DTT, 10% glycerol) and stored at -80 °C.

4.E.2 Pull-down analysis

Pull down experiments between Vif constructs and the Elongin BC complex were conducted by immobilizing 1 nmol of each Vif variant on Ni-NTA agarose. The Vif immobilized Ni-NTA agarose was incubated with 3 nmol of the Elongin BC complex for 30 minutes at 4 °C. After the incubation period, reactions were washed 7 times with 1 mL of Vif buffer (20 mM MOPS, 150 mM NaCl, 1 mM DTT, 10% glycerol). Reactions were then analyzed with SDS-PAGE and visualized by coomassie staining. Bands were then analyzed with the assistance of Jaylene Ollivierre (Northeastern University) with GE Healthcare Image Quant TL 1D software and the percentage of Elongin C bound to each Vif construct was determined and normalized to full length wt Vif.
4.E.3 Intact HX MS analysis

Hydrogen exchange reactions with Elongin BC and HIV-1 Vif were conducted by incubating the Elongin BC complex (4.00 μM) with or without a four fold excess of Vif (16 μM) at 4 °C for 30 minutes prior to deuterium labeling. Protein mixtures were then diluted 10-fold into 20 mM MOPS, 150 mM NaCl, 1 mM DTT (pD 7.0), D₂O at 25 °C for the indicated times. The percentage of Elongin BC bound to Vif during the hydrogen exchange reactions diluted in D₂O was calculated to be approximately 68% based on the Elongin BC and Vif diluted concentrations of 0.36 μM and 1.45 μM. When Vif was studied in the presence of an excess of Elongin BC, the above ratios were switched (Vif 4.00 μM, and Elongin BC 16 μM respectively). Reaction mixtures were quenched by lowering the pH to 2.6 with 0.8 M guanidine hydrochloride, 0.8% formic acid. Quenched samples were then analyzed as described in Chapter 2.C and Appendix III. Deuterium levels were not corrected for back exchange and are therefore reported as relative deuterium levels (Zhang, 1996).

For reactions with the Elongin BC complex and the Vif₁₃₅₋₁₅₈ peptide, the peptide (4.00 μM) was incubated with or without a four fold micro molar excess of the Elongin BC complex (16 μM). The percentage of Vif₁₃₅₋₁₅₈ peptide bound to the Elongin BC complex during the hydrogen exchange reactions diluted in D₂O was calculated to be approximately 95%.

For Elongin BC titrations with HIV-1 Vif, each Vif variant was titrated into a fixed concentration of Elongin BC (3.22 μM). The Elongin BC:Vif mixtures were incubated at 4 °C for 30 minutes before labeling. The mixtures were then incubated in a 10 fold excess of D₂O at 25 °C for 10 seconds. Protein mixtures were then quenched and analyzed as stated above. The percentage of Elongin C bound and unbound was determined by fitting the area under a charge state of Elongin C with a Gaussian distribution using the PeakFit® program (Systat, San Jose,
CA). The percentage of Elongin C bound was plotted versus Vif concentration (µM) and the data were fitted using a sigmoidal equation with Sigma Plot® software (Systat Software Inc, San Jose, CA). The reported $K_d$ values (Vif concentration that resulted in 50% Elongin C bound) were averaged values obtained from duplicate experiments.

### 4.E.4 Peptide HX MS analysis

Hydrogen exchange reactions for Elongin BC with and without Vif were performed as indicated above. Samples were digested and analyzed as described in Chapter 2.C and Appendix III.

### 4.E.6 Circular dichroism measurements

For circular dichroism measurements of Vif$_{135-158}$, the peptide was dissolved into 50 mM sodium phosphate to a concentration of 500 µM. Vif$_{135-158}$ (50 µM) samples were prepared with increasing concentrations of TFE and analyzed as described in Chapter 3.E.3. Deconvolution of CD spectra and estimation of secondary structure was conducted using the K2D2 software (Iratxeta, 2007).

### 4.F References


CHAPTER 5
Design and Synthesis of Peptide Inhibitors Targeting the Vif:Elongin BC Complex

5.A Introduction: inhibiting the Vif:Elongin BC complex

The work presented in Chapter 4 describes a peptide encompassing the Vif BC box associating with recombinant Elongin BC. These observations prompted an HX MS screen of Vif peptide sequences from the AIDS reagent program (AIDS reagent program, 2010) as potential inhibitors of the Vif:Elongin BC interaction. The work presented in this Chapter describes the design, screening, and synthesis of Vif$_{BC}$ box peptides as competitive inhibitors of full-length Vif:Elongin BC complex in vivo. Formation of the Vif:Elongin BC complex is essential for the poly-ubiquitylation of APOBEC3G as illustrated in Figure 5.1A and previously described in Chapter 4. A cartoon depiction of how a peptide could be used to inhibit the formation of the Vif:Elongin BC complex is illustrated in Figure 5.1B. If peptides or small molecules could be designed and synthesized that could bind to Elongin BC, they would prevent Vif from binding and directing APOBEC3G for poly-ubiquitylation and subsequent degradation. The antiviral activities of APOBEC3G would then disrupt further HIV-1 replication. The work presented illustrates the feasibility of designing, screening, and synthesizing Vif:Elongin BC inhibitors based on the Vif BC box sequence.
Figure 5.1 Inhibiting the Vif:Elongin BC complex. (A) Assembly of the Elongin BC dependent-E3 ligase complex is essential for APOBEC3G degradation (Mehle, 2004). (B) Inhibition of the Vif:Elongin BC interaction with peptides or small molecule drugs would disrupt APOBEC3G degradation and serve as a potential HIV therapy.
5.B Screening of Vif peptides from AIDS reagent program

5.B.1 Vif peptides binding to the Elongin BC complex

While the results of Vif\textsubscript{135-158}:Elongin BC binding were enlightening, the results did not indicate the minimal peptide sequence required for Elongin BC binding that could potentially be used as templates for inhibitors. A series of Vif peptides (15 residues in length) were obtained from the AIDS reagent program (AIDS reagent program, 2010) to screen for Elongin BC binding. Table 5.1 lists all of the Vif peptides screened. A cartoon representation of the Vif peptides is shown in Figure 5.2A. The peptides are labeled P1-6. P1 was chosen to serve as a negative control for Elongin BC binding as P1 is located in a region of Vif that is not implicated in the Vif:Elongin BC interaction, while the overlapping P2-P6 peptides span the Vif BC box. HX MS was used to screen for Elongin BC binding as Elongin C undergoes a significant conformational change when bound to SOCS ligands. The Elongin BC complex was incubated with a 2-fold molar excess of each Vif peptide and the reaction mixtures labeled for 1 minute. The 1 minute exchange time was chosen because there was large difference in deuteration between Elongin C as compared to 10 seconds bound and unbound as illustrate in Chapter 4, Figure 4.6. The controls for the binding experiment are shown in Figure 5.2B where the deuteration of Elongin BC alone served as the unbound control and the deuteration of Elongin BC bound to Vif\textsubscript{135-158} served as the bound control. The results in Figure 5.2B demonstrate that for the +9 charge state of Elongin C, the unbound higher deuterated form was observed at an m/z value of approximately 1225 (blue distribution), while the bound less deuterated form was observed at 1222 m/z (red distribution). These results illustrate how HX MS can be used to screen for Elongin BC binding peptides. The AIDS reagent Vif peptides were then assayed using HX MS and the results shown in Figure 5.2C. Incubation of Elongin BC with most of the Vif
<table>
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<th>Stop position</th>
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</thead>
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<td>1807</td>
<td>81</td>
<td>95</td>
</tr>
<tr>
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<td>P2</td>
<td>1541</td>
<td>137</td>
<td>151</td>
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<tr>
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<tr>
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<td>P6</td>
<td>1632</td>
<td>153</td>
<td>167</td>
</tr>
</tbody>
</table>

Table 5.1 Vif AIDS reagent program peptides. Shown are the Vif peptides screened for Elongin BC binding. The amino acid sequence, peptide designation, molecular weight, and amino acids numbers each peptide encompasses is indicated.
Figure 5.2 Screening of Vif peptide library for Elongin BC binding. (A) cartoon representation of the different peptides screened. The BC box helix is shown and the peptide capable of binding Elongin BC highlighted in green. Peptide are also described in Table 5.1. (B) Experimental controls for binding assay. Deuteration results for the +9 charge state of Elongin C are shown after 1 minute in the absence and presence of the Vif_135-158 peptide. The unbound distribution (higher mass) is shown in blue while the bound distribution (lower mass) is shown in red. (C) Binding results for the Vif peptides shown in (A) to the Elongin BC complex.
peptides resulted in Elongin C deuteration values consistent with the unbound form suggesting that these peptides did not interact with the Elongin BC complex. The only peptide that was capable of decreasing the deuteration of Elongin C was P3. The P3 peptide contains the residues that have been shown in the Vif<sub>140-156</sub>:Elongin BC crystal structure (Stanley, 2008) to form the BC box helix (illustrated in Figure 5.2A). Interestingly, the remainder of the peptides screened lacked a portion of the BC box helix. This is illustrated in the difference between the binding P3 peptide as compared to the non-binding P4 peptide which is lacking only one amino acid of the BC helix (serine 144). These results indicate that the minimum sequence requirement for Vif to bind Elongin BC is likely the BC box helix encompassing residues 144-155. Although it might be thought that the binding results obtained for the peptide library were to be excepted based on the Vif:Elongin BC X-ray structure, it is important to note that the minimum length Vif sequence required for Elongin BC binding has not been previously reported. Such a study was necessary to determine the minimum sequence required for binding so that inhibitor peptides would be made long enough to ensure that binding function would be retained.

5.B.2 A Vif<sub>BC box</sub> peptide capable of displacing a preloaded Vif:Elongin BC complex

After screening the peptides from the AIDS reagent program and identifying a peptide (P3) that was capable of binding Elongin BC, the P3 peptide (which encompasses the Vif<sub>BC box</sub>) was assayed for inhibitory activity towards Vif:Elongin BC binding. An observable change in Vif conformation (between bound and unbound) needed to be observed with HX MS to probe inhibition of Vif:Elongin BC binding. The HX MS results presented in Chapter 4 showed that no significant conformational changes occurred for full-length Vif upon binding the Elongin BC complex and therefore could not be used as a probe to test peptide inhibitors via HX MS. Unlike
full-length Vif, the Vif\textsubscript{135-158} peptide did undergo a significant conformational change upon Elongin BC binding and was therefore used as a probe for peptide inhibition. This concept is illustrated in Figure 5.3 where Vif\textsubscript{135-158} was pre-incubated with Elongin BC. When the Vif\textsubscript{135-158}:Elongin BC mixture was labeled for 10 seconds in D\textsubscript{2}O, The Elongin BC bound form Vif\textsubscript{135-158} displayed a centroid m/z value at approximately 1139 m/z. The deuteration of Vif\textsubscript{135-158} in the unbound form resulted in a centroid m/z value at approximately 1143 m/z. If a potential peptide inhibitor (i.e. P3) of the Vif\textsubscript{135-158}:Elongin BC complex was titrated and the peptide displaced Vif\textsubscript{135-158} from Elongin BC, there would be change in the observed centroid m/z value for Vif\textsubscript{135-158}. The results of the P3 titrations are shown for the +3 charge state of Vif\textsubscript{135-158} in Figure 5.4. Panel (A) illustrates the experimental controls for Vif\textsubscript{135-158} bound to Elongin BC (red distribution), unbound (blue distribution), and bound to Elongin BC in the presence of the non-binding peptide P1. The bound form of Vif\textsubscript{135-158} displayed more protection from deuteration than the unbound form by approximately 10 Da, making the two forms easily distinguishable. The presence of a 20-fold molar excess of P1 to Vif\textsubscript{135-158} did not result in a change in Vif\textsubscript{135-158} deuteration indicating that P1 did not bind to Elongin BC and displace Vif\textsubscript{135-158}. The P1 sequence is from a region in Vif that has no known Elongin BC binding activity and the results suggest P1 did not disrupt the binding between Vif\textsubscript{135-158} and the Elongin BC complex. The P3 peptide was tested using the HX MS assay and the raw spectra are shown in Figure 5.4B. At the lowest concentration of P3 (3 µM), there was no observable change in deuteration for the Vif\textsubscript{135-158} peptide indicating that Vif\textsubscript{135-158} remained bound to Elongin BC. As the concentration of P3 increased, the deuteration of the Vif\textsubscript{135-158} peptide changed as the unbound deuteration m/z peak of 1143 m/z appeared. This peak became larger as the concentration of P3 was increased suggesting that P3 was binding to Elongin BC preventing Vif\textsubscript{135-158} from associating. The
Figure 5.3 Vif<sub>135-158</sub> displacement assay. Cartoon representation of the assay used to test peptide displacement of Vif<sub>135-158</sub> from the Elongin BC complex.
Figure 5.4 Inhibition of the Vif135-158:Elongin BC complex with a Vif BC box peptide. (A) Experimental controls showing Vif135-158 bound to Elongin BC (blue) and unbound (red). (B) Titration of the Elongin C binding peptide P3 to a Vif135-158:Elongin BC complex mixture. (C) Percentage of Vif135-158 bound as a function of P1 and P3 concentration. Data were fitted using an exponential decay equation with Sigma Plot® software (Systat Software Inc, San Jose, CA).
percentage of bound Vif_{135-158} to Elongin BC was calculated by fitting the area under each peak in (B) and graphed as a function of P3 concentration. The results for the same titration using the P1 peptide are also shown. The graph shows that as the concentration of P3 was increased, the percentage of bound Vif_{135-158} decreased. These results indicate that the P3 peptide displaced Vif_{135-158} from the Vif_{135-158}:Elongin BC complex resulting in the appearance of the more deuterated unbound form of Vif_{135-158}. One interesting observation from the graph in Figure 5.4C is that even at high concentrations P3 did not completely displace the Vif_{135-158}:Elongin BC interaction. This observation can likely be attributed to the higher affinity of Vif_{135-158} as compared to P3 for the Elongin BC complex as Vif_{135-158} is longer than the P3 sequence and contains more residues that make contact with Elongin C. Even thought P3 did not completely abolish Vif_{135-158}:Elongin BC complex formation, the results presented demonstrate that the Vif BC box helix region (P3) can be used as a template to design peptide competitive inhibitors against the Vif_{135-158}:Elongin BC complex.

5.C Designing and synthesizing stapled Vif_{BC} box peptides

5.C.1 Advantages of stabilizing α-helical peptides with hydrocarbon stapling

The results in the previous Section demonstrate that the Vif BC box region can be used to inhibit the binding of Vif_{135-158} to Elongin BC. Even though the results presented are promising, there are major limitations to employing peptides as therapeutic agents. One major difficulty in using peptides as inhibitors is that when small segments of proteins are selected to use as inhibitors, they usually do not retain any structure in the absence of the entire protein. The lack of structure results in reduced cell permeability and makes the peptides sensitive to proteolytic
digestion (Kim, 2011; Bird, 2008; Henchey, 2008). Therefore, the implementation of alternative strategies at synthesizing Vif peptide inhibitors was explored.

One methodology that has been previously employed to stabilize α-helical peptides and address the issues above is known as “peptide stapling” which is illustrated in Figure 5.5A and B. Peptides that normally adopt a α-helical conformation can be synthesized and a staple incorporated through the introduction of two (S) α,α-disubstituted pentenyl alanine residues four residues apart from each other. The staple consists of a macrocyclic bridge formed between the two modified amino acids using the ruthenium-mediated ring closing olefin metathesis reaction (Blackwell, 1998; Bird, 2008; Walensky, 2004). Peptide stapling has been successfully employed in the design and synthesis of stapled peptides whose targets include the re-activation of the p53 pathway, BH3 activation of apoptosis in cancer cells, inhibition of HIV particle assembly, and inhibition of the NOTCH signaling (Bernal, 2007; Walensky, 2004; Zhang, 2011; Bird, 2010; Moellering, 2009). Numerous reports have demonstrated that peptide stapling increases α-helicity, imparts superior proteolytic resistance, extends in vivo half-lives, and allows endocytic cell penetration (Bird, 2010; Madden, 2011, Kim 2009;). Therefore, hydrocarbon stapling was tested to determine if this methodology could be employed in making Vif:Elongin BC inhibitors.

5.C.2 Design considerations for synthesis of stapled Vif<sub>BC box</sub> peptides

There are several different stapling systems currently available to produce stapled peptides including the \(i+3\), \(i+4\), and \(i+7\) stapling systems [for a review of stapling systems see (Kim, 2011; Henchey, 2008)]. The system chosen for this work utilizes the \(i+4\) staple system which staples two α,α-disubstituted pentenyl alanine residues together that are one helical turn
Figure 5.5 Hydrocarbon stapling of helical peptides. (A) Grubbs catalyst mediated ring closing metathesis of peptides containing (S) α,α-disubstituted pentenyl alanine. (B) Benefits of using stapled peptides as therapeutic agents.
apart. One aspect that is essential when designing stapled peptides, regardless of the stapling system chosen, is the location where the α,α-disubstituted amino acids are introduced. The hydrocarbon macrocyclic bridge consists of an eight carbon chain that extends from one side of the helix. A requirement for stapled peptides to remain biologically active is that this bridge has to be placed in a position that will not interfere with any biological functions such as protein binding. The macrocycle is usually placed on the opposite side of the protein:protein interface so that the native side chains that participate in binding are not disturbed. In the design of Vif:Elongin BC peptide inhibitors, the Vif140-156:Elongin BC X-ray structure was employed for considerations as shown in Figure 5.6 (Stanley, 2008). The structure shows the Vif BC box (red) bound to Elongin C (yellow). The positions that were chosen for replacement with α,α-disubstituted pentenyl alanine are shown in blue which consisted of tyrosine147, and alanine151. These residues were chosen because they were on the opposite face of the Vif:Elongin BC interacting interface (rotated view Figure 5.6) and also have been shown that substitution does not alter Vif:Elongin BC binding (Mehle, 2004). The sequence that was chosen as a template for the Vif stapled peptides was Vif138-157. The extra residues (absent in P3, 138-140 and 156-158) were included on the N-terminus to provide spacing between the Vif BC helix and any N-terminal tags that were added (such as FITC) to ensure that the peptide still bound Elongin BC. This peptide template will be referred to in the rest of this Chapter as the Vif_{BC} box.

5.C.3 Synthesis of stapled Vif_{BC} box peptides

Having chosen the residues for replacement with α,α-disubstituted pentenyl alanine and the sequence to be synthesized, peptides were produced using solid phase peptide synthesis as described in Figure 5.7 using an automated peptide synthesizer (see Material and Methods).
Figure 5.6 Design considerations for stapled Vif_{BC box} peptides. Shown is the Vif_{140-156}:Elongin BC X-ray structure (Stanley, 2008). The residues chosen for replacement with $\alpha,\alpha$-disubstituted pentenyl alanine are highlighted in blue. A rotated view of the Vif_{140-156}:Elongin BC X-ray structure is shown and the ligand binding interface shown.
Figure 5.7 Synthesis of Vif peptides. Vif peptides were produced using solid phase peptide synthesis as illustrated above. Adapted from (Bird, 2008).
Fmoc amino acids were coupled by first deprotecting Fmoc protected amino acids onto the growing peptide chain with 20% piperdine, followed by coupling with the next amino acid in the presence of the coupling activator HCTU and the activator base DIEA. This process was cycled for each amino acid introduced. Peptides that were FITC labeled had an additional N-terminal beta-alanine included to prevent cyclization and FITC loss during the final cleavage reaction (Jullian, 2008). After introduction of the $\alpha,\alpha$-disubstituted pentenyl alanines, the ring closing metathesis reaction was conducted on resin bound peptides by incubation with the 1st generation Grubbs catalyst [for a review on Grubbs catalyst mediated ring closing metathesis see (Binder, 2008; Grubbs, 2006)]. Peptides were either cleaved from the resin with TFA or FITC labeled prior to cleavage. Table 5.2 describes all Vif peptides produced.

5.D Characterization of stapled Vif<sub>BC</sub> box peptides

5.D.1 Mass analysis of Vif<sub>BC</sub> box peptides

Peptides were mass analyzed after synthesis, stapling, and modification as illustrated in Figure 5.7. Representative spectra of the non-FITC labeled peptides are shown in Figure 5.8A for the unmodified sequence (i), modified unstapled (ii), and the modified stapled (iii). The major charge species that dominate each spectrum corresponds to the indicated peptide showing that the Vif peptides were successfully synthesized and purified. Mass analysis of the modified peptides revealed that the stapling reaction was successful which is indicated by the loss of ethylene as shown in spectra in Figure 5.8B.
Table 5.2 Vif synthetic peptides. Shown are the Vif peptide sequences and any modifications made. The Fluorescein isothiocyanate (FITC) tag is shown in green, and the position where α,α-disubstituted pentenyl alanine was substituted is indicated by the $. The Vif$_{BC}$ box peptides that did not contain any modifications to their amino acid sequences are labeled unmodified. The Vif$_{BC}$ box peptides that contained α,α-disubstituted pentenyl alanines that were unstapled are labeled modified unstapled. The Vif$_{BC}$ box peptides that contained α,α-disubstituted pentenyl alanines that were stapled were are labeled modified stapled.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Peptide description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHNKVGSLQYLALAALITPK</td>
<td>FITC−β-ala-GHNKVGSLQYLALAALITPK</td>
</tr>
<tr>
<td>GHNKVGSLQ$LAL$ALITPK</td>
<td>FITC−β-ala-GHNKVGSLQ$LAL$ALITPK</td>
</tr>
<tr>
<td>GHNKVGSLQ$LAL$ALITPK</td>
<td>FITC−β-ala-GHNKVGSLQ$LAL$ALITPK</td>
</tr>
</tbody>
</table>
Figure 5.8 ESI-mass spectra of Vif<sub>BC box</sub> peptides. (A) (i) 200 pmols of unmodified Vif<sub>BC box</sub>, (ii) Vif<sub>BC box</sub> modified unstapled, and (iii) Vif<sub>BC box</sub> modified stapled. (B) +2 charge state of Vif<sub>138-157</sub> modified unstapled, and Vif<sub>138-157</sub> modified stapled showing that successful ring closing metathesis results in a reduction in mass that corresponds to the loss of ethylene. The pink and green peaks in (A) (ii and iii) correspond to the peaks in (B).
5.D.2 Binding of Vif$_{\text{BC box}}$ peptides to Elongin BC

Binding between the Vif$_{\text{BC box}}$ peptides and Elongin BC was tested to ensure that the different modifications including stapling did not interfere with complex formation. Binding of the Vif peptides to Elongin BC was assayed as described above for the AIDS reagent program peptides. The results are shown in Figure 5.9 for the +9 charge state of Elongin C. The deuteration of the Elongin BC complex alone was used as an unbound control which is indicated by the blue distribution. The deuteration of Elongin C upon incubation with the Vif$_{\text{BC box}}$ unmodified peptide was suppressed as compared to the unbound form indicating that the unmodified sequence interacted with Elongin BC. The deuteration of Elongin C in the presence of the Vif$_{\text{BC box}}$ modified unstacked peptide was similar to the unbound form indicating that the presence of the $\alpha,\alpha$-disubstituted pentenyl alanine residues hindered binding to Elongin BC. This observation can likely be explained by the effects of the modified amino acids preventing the Vif$_{\text{BC box}}$ from folding into a conformation competent to bind Elongin BC. Another explanation for these observations could be that the unstacked peptide formed oligomers in solution, due to the presence of the hydrophobic un-metathesized $\alpha,\alpha$-disubstituted amino acids, which could prevent binding to the Elongin BC complex. Interestingly, the deuteration for Elongin C in the presence of the Vif$_{\text{BC box}}$ modified stapled peptide was similar to the bound form of Elongin C suggesting that the hydrocarbon stapled peptide was capable of binding to Elongin BC. The results for the stapled peptide in Figure 5.9 demonstrate that the hydrocarbon staple did not interfere with complex formation and that the stapled Vif peptide adopts a conformation competent to bind Elongin BC.
Figure 5.9 Binding of Vif\textsubscript{BC box} peptides to Elongin BC. Shown are the deuteration results for the Elongin C +9 charge state upon incubation with the different Vif peptides. The bound form of Elongin C is highlighted in red and the unbound form in blue.
5.D.3 CD analysis of Vif\textsubscript{BC} box peptides

Having assayed the binding between Elongin BC and the Vif\textsubscript{BC} box peptides, CD was utilized to determine if the amino acid modifications and hydrocarbon stapling had any influence on the secondary structure of the Vif peptides. Vif peptides were dissolved in phosphate buffered saline and analyzed as described in Chapter 4. The CD spectra for the Vif\textsubscript{BC} box peptides are shown in Figure 5.10A. The CD trace of the unmodified sequence is shown in blue and is indicative of irregular secondary structure as indicated by the minimum below 200 nm. The introduction of the \(\alpha,\alpha\)-disubstituted pentenyl alanine residues shifted the CD spectrum of the modified unstapled peptide to two minima at approximately 205 and 222 nm (dotted trace). These results suggest that introduction of \(\alpha,\alpha\)-disubstituted pentenyl alanine results in the peptide adopting a more alpha helical conformation. The CD trace of the stapled peptide is shown in red which has similar minima to that of the unstapled peptide, however, the amplitude of the CD spectrum for the stapled peptide is much greater indicating an increase in alpha helicity upon hydrocarbon stapling. The \% alpha helical content of each peptide was calculated [see (Forood, 1993) for calculation] and is shown in Figure 5.10B. The results illustrate that introduction of the \(\alpha,\alpha\)-disubstituted pentenyl alanine residues increased the \% alpha helicity of the modified unstapled peptide by 2-fold as compared to the unmodified peptide. The stapled peptide had a 3.7-fold increase in helicity as compared to the unmodified peptide.

5.D.4 HX MS analysis of Vif\textsubscript{BC} box peptides

HX MS analysis was utilized to further probe the conformational effects of the \(\alpha,\alpha\)-disubstituted amino acid modification and hydrocarbon stapling on the Vif\textsubscript{BC} box peptides. The results are shown in Figure 5.11 for the Vif\textsubscript{BC} box peptides. The raw spectra for the +2 charge state
Figure 5.10 CD analysis of Vif<sub>BC</sub> box peptides. (A) Shown are the CD spectra for Vif<sub>BC</sub> box unmodified (blue), Vif<sub>BC</sub> box modified unstapled (dotted line), and Vif<sub>BC</sub> box modified stapled (red). (B) Percent alpha helicity of the Vif<sub>BC</sub> box peptides. Color is the same for Panel A. % helicity was calculated using the shown equation [see (Forood, 1993) for complete description of calculation].
are shown in Figure 5.11A for the Vif\textsubscript{BC} box unmodified peptide (i) and the modified stapled (ii). The undeuterated spectra are shown in grey and the 10 second labeling time is shown in blue for the unmodified peptide and red for the stapled peptide. This time point was selected to highlight the greatest difference in deuteration. The undeuterated masses for each peptide are different as indicated by the different m/z scales; however, all spectra are shown at a fixed m/z window of 11.2 m/z to allow for comparison. The Vif\textsubscript{BC} box unmodified peptide was rapidly deuterated in 10 seconds as indicated by 15 Da mass shift from the undeuterated sample. The Vif\textsubscript{BC} box unmodified peptide has a possible maximum deuteration of 16 amide hydrogens and after only 10 seconds in D\textsubscript{2}O, 15 of these positions are exchanged (corrections for back exchange were not performed). The HX MS observations for the Vif\textsubscript{BC} box unmodified peptide are in agreement with the CD results and suggest that the Vif\textsubscript{BC} box adopts an unfolded conformation in solution. The HX MS results for the stapled peptide are different than the unmodified peptide in that the stapled version only exchanged 9 amide hydrogens after 10 seconds as shown in Figure 5.11A (ii). Hydrocarbon stapling of the Vif\textsubscript{BC} box protects 6 amide positions from exchange which is consistent with the idea that the staple locks the peptide in an alpha helical conformation which is protected from amide exchange.

**5.D.5 Cellular uptake of Vif\textsubscript{BC} box peptides**

Hydrocarbon stapling of small peptides has been shown to promote endosomal uptake (Bird, 2008; Walensky, 2004; Kim, 2009; Walensky, 2006). To test the ability of the stapled Vif\textsubscript{BC} box peptide to enter cells, confocal fluorescence microscopy and fluorescence activated cell (FACS) sorting were utilized to test peptide uptake. For peptide uptake experiments, the stapled Vif\textsubscript{BC} box peptide was incubated with HeLa cells at concentrations ranging from 1-5 μM for
Figure 5.11 HX MS analysis of Vif<sub>BC<sub>box</sub> peptides. (A) Mass shifts for 10 seconds of deuteration for the Vif<sub>BC<sub>box</sub> unmodified peptide (i) and the modified stapled Vif<sub>BC<sub>box</sub> peptide (ii). The undeuterated mass of each peptide differs, however, the m/z scales are the same range (11.2 m/z range) to allow for comparison. (B) Deuterium uptake curve for the different Vif peptides. The error of intact HX MS measurements was ± 2 Da.
various amounts of time. Representative confocal fluorescence images of live HeLa cells are shown in Figure 5.12A. Shown are the Brightfield and fluorescence images for HeLa cells treated with DMSO and the FITC-labeled stapled Vif$_{BC}$ box peptide. The fluorescence image of the DMSO treated cells shows very low fluorescence as compared to the peptide treated cells which displayed localized fluorescent areas. These results indicate that upon incubation of HeLa cells with the FITC-labeled peptide, more focused fluorescence was observed indicating that the peptide efficiently entered cells.

An orthogonal technique to confocal fluorescence microscopy is FACS. FACS is capable of sorting cells based on their relative fluorescence and is advantageous in studying the uptake of FITC-labeled peptides because trypsin digestion is used prior to FACS analysis. Trypsin digestion of cell samples serves two purposes: to liberate cells from culture dishes and to remove any surface bound FITC-labeled peptide [see (Richard, 2003) for example] which is one concern when studying peptide uptake. FACS analysis was conducted on HeLa cells incubated with the FITC-labeled stapled Vif$_{BC}$ box peptide and subjected to trypsin digestion. FACS results are shown in Figure 5.12B for cells incubated with DMSO (green line), FITC-labeled stapled peptide for 15 minutes (blue line), and FITC-labeled stapled peptide for 1 hour (red line). The results show that upon incubation with the FITC-labeled stapled peptide, the cell distribution shifted to the right with longer incubation times which corresponds to an increase in fluorescence. The FACS results are in agreement with the confocal fluorescence microscopy images obtained and suggest that the FITC-labeled stapled Vif$_{BC}$ box peptide efficiently entered HeLa cells.
Figure 5.12 Cellular uptake of the Vif$_{BC}$ box stapled peptide. (A) confocal fluorescence microscopy of HeLa cells incubated with a DMSO control or 1 µM Vif$_{BC}$ box modified stapled peptide. Shown are Brightfield images and the corresponding fluorescence images. (B) FACS analysis of Vif$_{BC}$ box modified stapled peptide uptake into HeLa cells. Fluorescence images were acquired with the assistance of Dr. Tatyan Chernenko (Northeastern University), and FACS analysis with the assistance of Alima Sulemana (Northeastern University).
5.D.6 HIV-1 inhibition by a VifBC box peptide

The results described above show the ability to synthesize, purify, and test *in vitro* VifBC box peptides as Vif:Elongin BC binding inhibitors. The ultimate test for the described VifBC box peptides would be to see if they exhibited antiviral activity against HIV-1. VifBC box peptides were given to Dr. Jerrod Poe (University of Pittsburgh) to test for antiviral activity in an HIV-1 replication assay. Inhibition of viral replication was tested using non-permissive (cells that express APOBEC3G, Vif is needed for viral replication, see Chapter 1) CEM T4 cells which were grown in the absence or presence of varying concentrations of the FITC-labeled unmodified VifBC box peptide. The CEM T4 cells were then infected with the HIV-1 NL4-3 virus and viral replication monitored as determined by the levels of the HIV-1 viral component p24. Preliminary results for the FITC-labeled unmodified peptide are shown in Figure 5.13. The levels of p24 from CEM T4 cells untreated with peptide and infected with HIV-1 are shown by the blue line and were approximately 42000 pg/mL. CEM T4 cells untreated and uninfected did not have any measurable p24 levels as indicated by the green line. The p24 levels from CEM T4 cells treated with the Vif peptide and infected are shown by the red line in Figure 5.13. Interestingly at low concentrations of the FITC-labeled unmodified VifBC box peptide, p24 levels were similar to untreated cells infected with HIV-1. An observable decrease in p24 levels occurred at peptide concentrations above 1 μM indicating that the Vif peptide exhibited antiviral activity at concentrations above 1 μM. Cell viability was also monitored (data not shown) for all experimental conditions and remained within 98-100% viable indicating that the Vif peptide was not cytotoxic. The preliminary HIV replication results are promising and indicate that peptides designed around the VifBC box could be utilized as anti-HIV treatments.
Figure 5.13 Inhibition of HIV-1 replication with Vif$_{BC\text{ box}}$ peptide. Data acquired and provided by Dr. Jerrod Poe (University of Pittsburgh). CEM T4 cells were infected with the laboratory HIV-1 viral strain NL4-3 in the absence (blue line) or presence (red) line of a Vif$_{BC\text{ box}}$ unmodified peptide at varying concentrations and the levels of the HIV-1 viral component p24 detected using ELISA. Uninfected untreated cells are shown (green). Errors are reported for 4 replicate experiments.
The results presented in this Chapter provide the foundation for future work on Vif:Elongin BC based inhibitors. Some challenges that remain for this research are: (1) to determine if hydrocarbon stapling is necessary for the inhibition of the Vif:Elongin BC complex, (2) determine the mechanism by which the Vif$_{BC}$ box peptides enter cells and where they localize, and (3) to validate biochemically that the Vif$_{BC}$ box peptides are inhibiting the Vif:Elongin BC interaction (without cytotoxicity) resulting in loss of APOBEC3G degradation. Conducting these experiments and controls would further validate the Vif:Elongin BC axis as a potential target for anti-HIV therapy.

5.E Summary

The HIV-1 Vif:APOBEC3G axis is an attractive target for antiviral therapy as inhibition of APOBEC3G degradation allows the immune system to directly combat infection. Inhibiting the many interactions between Vif and the E3 ligase machinery, including Vif:Elongin BC binding, could block APOBEC3G degradation. The work presented in this Chapter describes the design, synthesis, testing of Vif peptide inhibitors based on the Vif$_{BC}$ box helix from the Vif SOCS box. The results described in Sections 5.C and D for hydrocarbon stapling demonstrate that the Vif$_{BC}$ box can be modified and still retain Elongin BC binding activity illustrating the robustness of Vif$_{BC}$ box peptides as potential antiviral therapies.

One potential concern with using peptides to bind Elongin BC and block Vif is that any cellular proteins that bind Elongin BC would also be inhibited leading to cytotoxic effects. The preliminary cell viability data (data not shown) which correspond to the HIV-1 replication assay described in Section 5.D.6 suggest that the unmodified Vif$_{BC}$ box peptide inhibited viral replication with no cytotoxic side effects. Several other observations have been made and
reported in the literature, that suggest the complex formation between Vif and Elongin BC is different than for Elongin BC with cellular proteins. The work in Chapter 4 and in the literature (Bergeron, 2010; Wolfe, 2010) indicate that Vif has the ability to interact with the Elongin BC complex in the absence of other cellular components. The association of cellular Elongin BC binding proteins differs in that they require co-expression \textit{in vivo} or the presence of folding chaperonins to associate with Elongin BC as reported for the SOCS family and VHL (Babon, 2009; Feldman, 1999; Babon, 2008). These observations raise the possibility that Vif peptide inhibitors might selectively block Vif binding to Elongin BC, while cellular proteins could still be loaded into a functional Elongin BC complex through a separate mechanism(s). Regardless of the obstacles in developing Vif\textsubscript{BC} box peptide based inhibitors, the work presented here provides the foundation for future development of Vif peptide based inhibitors towards the Vif:Elongin BC complex. Future work could include the screening of different lengths of Vif sequence for stapled peptides along with different hydrocarbon stapling systems. Such investigation might lead to the discovery of more potent Vif:Elongin BC inhibitors.

5.F Materials and methods

5.F.1 Peptide synthesis

Vif peptides were synthesized on a 100 µmol scale using standard 9-fluorenylmethoxycarbonyl (Fmoc) peptide chemistry on NovaPEG Rink amide resin using a CEM Liberty 9008005 microwave peptide synthesizer. Subsequent chemical modifications were carried out manually. Ring closing metathesis was conducted on resin bound peptide by incubation with the Grubbs 1\textsuperscript{st} generation catalyst at a 1:5 catalyst:peptide molar ratio. Ring closing metathesis was conducted twice for one hour incubations in dichloroethane. FITC
labelling of peptides was conducted on resin for 12 hours by incubation of peptides with a 3-fold molar excess of FITC and 5-fold molar excess of DIEA. Peptides were cleaved from resin using 95% TFA/2.5% TIS/2.5% H₂O and precipitated using cold diethyl ether. Peptides were purified on a Varian ProStar HPLC using a Dynamax 21.4 X 250 mm 300Å C18 column eluted with water-acetonitrile containing 0.1% TFA as the mobile phase and UV monitoring at 220 nm. The peptide containing fractions were collected; solvent removed by lyophilization, and purified material stored at -80 °C. Peptides were synthesized with the assistance of Dr. Jason Marineau (DFCI).

The HIV-1 Consensus B Vif (15-mer) peptides were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus B VIF (15-mer) Peptides - Complete Set. Peptide information can be found in Table 5.1. The Vif₁₃₅-₁₅₈ peptide was obtained as described in Chapter 4.

5.F.2 HX MS analysis

Peptide binding to Elongin BC was assayed by incubation of Elongin BC with a 2-fold molar excess of the indicated peptides at 4 °C for 1 hour prior to labeling. Peptide:Elongin BC mixtures were then labeled using a 10-fold dilution of D₂O (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.0). Labeling reactions were quenched after 1 minute using 0.8% FA, 0.8 M GdnHCl, and mass analyzed as described in Chapter 2.C

Vif₁₃₅-₁₅₈ displacement was assayed by pre-incubating the Vif₁₃₅-₁₅₈ peptide with a 1.5-fold molar excess of Elongin BC at 4 °C for 1 hour. The potential peptide inhibitors (P1 and P3) were then added at various concentrations to the Vif₁₃₅-₁₅₈:Elongin BC mixture and incubated at
4 °C for 1 hour. Mixtures were labeled for 10 seconds; quenched, and analyzed as described above.

HX MS conformational analysis of Vif_{BC} box peptides was carried out by a 10-fold dilution of each peptide into D_{2}O for the indicated times; quenched, and mass analyzed as described above.

5.F.3 CD analysis

Circular dichroism measurements of Vif_{BC} box peptides were obtained by dissolving each peptide into 50 mM sodium phosphate to a concentration of 500 μM. Peptides were then diluted (50 μM) and analyzed as described in Chapter 3. The % alpha helicity for Vif peptides was calculated according to (Forood, 1993).

5.F.3 Confocal fluorescence microscopy

Cell culture for all confocal fluorescence microscopy experiments was conducted by Ellen Marcisisin (Northeastern University). HeLa cells (50-100 k) were seeded onto 25 mm x 2 mm CaF₂ windows and incubated overnight at 37 °C (5% CO₂) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. Cells were then washed and resuspended in non-FBS supplemented Dulbecco’s Modified Eagle’s Medium. FITC labeled peptide stocks were prepared by dissolving each peptide into 100% DMSO to a concentration of 1 mM. Concentrations of FITC labeled peptides were determined by UV absorption at 488 nm and calculated using the extinction coefficient 67000 M⁻¹ cm⁻¹ (Mitchell, 2000). The FITC labeled peptides were added to cells at a concentration of 1 μM and incubated for 2 hours at 37 °C. Cells were then washed extensively with PBS and imaged in PBS using a Confocal Raman Microscope (CRM200) from
WITec (Ulm, Germany). Fluorescence images were acquired with the assistance of Dr. Tatyana Chernenko (Northeastern University). Fluorescence images were obtained by using an excitation wavelength of 488 nm and monitoring emission at 518 nm.

5.F.4 FACS analysis

Cell culture for all FACS experiments was conducted by Ellen Marcisin (Northeastern University). HeLa cells (50-100 k) were seeded onto cell culture plates (35 x 10 mm) and incubated overnight at 37 °C (5% CO2) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. Cells were then washed and resuspended in non-FBS supplemented Dulbecco’s Modified Eagle’s Medium FITC labeled peptides were then added to cells at a concentration of 1 μM and incubated for the indicated times at 37 °C. Cells were then washed extensively with PBS and removed from the culture plates by incubation with a 1X trypsin-EDTA solution (ATCC, Manassas, VA). Cells were pelleted by centrifugation and resuspended in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. Cells were pelleted again and then resuspended in 1X PBS. FACS analysis was carried out on a Becton Dickinson FAC scan flow cytometer with the assistance of Alima Sulemana (Northeastern University).

5.F.5 HIV-1 replication assays

HIV-1 replication assays were conducted by Dr. Jerrod Poe (University of Pittsburgh). Briefly, CEM T4 cells were incubated with the indicated concentrations of Vif peptide and then infected with the HIV-1 NL4-3 virus. Viral replication was monitored by p24 ELISA as described in (Poe, 2009).
5.G References

AIDS reagent program (2010). The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 Consensus B VIF (15-mer) Peptides - Complete Set.


CHAPTER 6
Using the Vif:Elongin Complex and Fluorous Chemistry in the Development of a Continuous Flow Hydrogen Exchange System

6.A Introduction

The ability of HX MS to detect changes in protein conformation provides a powerful tool in structural biology. Examples of the utility of HX MS for protein conformational analysis are provided throughout this dissertation; however, there are several inherent limitations in HX MS methodology. A major requirement is the production/isolation of a desired protein system for analysis as well as manual sample handling and deuterium labeling. Protein over-expression and purification can be challenging and take months to be successful. Once isolated, proteins are then subjected to traditional HX analysis by manual dilution into D$_2$O, a process that can be error prone, and time consuming. Advancements in HX MS methodologies that would allow for the single step isolation of protein systems from complex mixtures, such as *E. coli* or mammalian cell lysates, and a platform that would allow for an automated flow HX exchange system would contribute to the advancement of the field. The work described in this Chapter illustrates the use of the Elongin BC complex as a model system to test an HX platform based on fluorous chemistry (fluorous chemistry and the fluorous effect will be described below). This platform directly addresses the limitations described above.
6.A.1 Utility of a fluorous capture system in HX MS

The ability to conduct HX of proteins on a surface would avoid conventional sample dilution techniques and allow for potential automation because D\textsubscript{2}O could be flowed directly over the surface. Such a system could be designed and built onto a micro-fluidic chip. The sample loading, washing, HX labeling, and elution could then be controlled by a computer.

There have been previous reports in the literature of utilizing flow chambers to conduct HX, however the proteins being analyzed are not retained on a surface and instead flowed through a deuterated buffer stream (Astorga-Wells, 2011; Pan, 2010). In order to conduct HX of a protein on a surface, the protein of interest has to be captured on the surface, retained during D\textsubscript{2}O incubation, and released after quenching for MS analysis. The problem with many tag/capture systems is that they do not tolerate the low pH conditions required of the HX quenching step. This makes multiple capture and release cycles not possible. The ability of the tag/capture system to tolerate HX quenching conditions and to conduct multiple capture and release cycles of the target protein is essential when developing such a system. Some important considerations in the development of a capture system are: the surface used for the support is (1) inert to chemical degradation under different buffer conditions (i.e. labeling and quench), (2) have low non-specific binding, and (3) not alter the conformation of the protein being analyzed.

One system that has recently been shown to fulfill the above requirements and utilized in HX experiments is the biotin-strepavidin tag/capture system (Ling, 2011). Another system that has not been explored for HX applications, but, utilized vigorously in other fields is based on the self associative properties of poly-fluorinated compounds known as the fluorous effect [see (Mandal, 2008; O’Neal, 2010; Neil, 2000; Lee, 2006) for examples]. The use of fluorous chemistry was thus chosen in the design, synthesis, and testing of a tag/capture flow HX MS
platform and is illustrated in Figure 6.1. Fluorous chemistry became popular following the seminal publication by Horváth and Rábai demonstrating the utility of perfluorocarbon solvents and the fluorous effect in chemical synthesis (Horváth, 1994). There are several advantages to using perfluorocarbon materials and surfaces which include: they are chemically inert over a wide range of different conditions, have relatively low non-specific binding, and have high affinity for other fluorinated substances (Chen, 2010). These properties make fluorous chemistry ideal in the development of an HX platform.

The system illustrated in Figure 6.1 requires a chemical probe specific for the target protein that is to be analyzed with HX MS. The probe can be a small molecule, peptide, and/or protein that associates with the target protein in solution. Covalently attached to the probe is a fluorous tag (a C$_8$F$_{17}$ or C$_6$F$_{13}$ tag) which tightly associates with a fluorinated surface. The fluorous surface that was chosen for the work described in this Chapter was fluorous coated silica beads packed into a column format. To capture the desired protein, the target is flowed over the fluorous probe loaded surface. After successful capture, the target protein is labeled by flowing D$_2$O over the surface (through the column) for the desired labeling time. After labeling, the HX reaction is quenched and target protein eluted from the probe by chemical denaturation. The eluted protein is either mass analyzed in its intact form or digested first with pepsin and analyzed as peptides.

### 6.A.2 The fluorous effect

The chemical properties of perfluorocarbons make the system illustrated in Figure 6.1 possible. The chemical properties of perfluorinated hydrocarbons differ significantly from hydrocarbons because of the fundamental differences between the C-F and C-H bonds. These
Fluorinated (C8F17 or C6F15) chemical Probe

- Protein
- Peptide
- Small molecule

Fluorinated species adhere via fluorous effect
(pH 7.0, target protein formulated buffer)

D2O reaction quenched after desired time,
target denatured and eluted
(pH ≤ 2.6, 8 M Guanidine HCl,
0.1% Formic Acid)

D2O added (flow) to column to probe target conformation
(pH 7.0, target protein formulated buffer)

MS Analysis of target protein
Intact or peptides

Intact or Pepsin digested

Column Re-equilibration

Target protein binds to chemical probe
(pH 7.0, target protein formulated buffer)

Target protein

Figure 6.1 The fluorous capture flow HX MS workflow. Illustrated is the use of fluorous capture using a fluorous labeled probe against a target protein. Once captured, the target protein is subjected to labeling through a continuous flow of D2O for desired labeling times. After labeling, the HX reaction is quenched and the target protein is eluted by denaturation for subsequent MS analysis.
differences lead to several unique properties of perfluorinated carbons: perfluorocarbons are chemically inert over a wide range of conditions (Cametti, 2011), and are difficult to solubilize in most aqueous and hydrocarbon solvents. Perfluorocarbons will dissolve in perfluorinated solvents, and are thus said to be fluorophilic. This phenomenon is known as the fluorous effect. Figure 6.2 illustrates the fluorous effect showing the separation of triphasic system of hydrocarbon, aqueous, and perfluorocarbon solvents (colored with dye). The phase separation illustrated in Figure 6.2 and fluorous effect can be attributed to the differences in the C-F and C-H bond polarizabilities. Cohesive London Dispersion forces, which arise from temporary induced dipoles, are greater in hydrocarbons because C-H bonds are more easily polarized than C-F bonds (Gottler, 2008). The fluorous effect can be explained more generally by the thermodynamic theory of non-electrolyte solutions for two non-polar molecules (substance 1 and 2) as described in Equation 6.1 (Lee, 2004; Scott, 1948).

\[
\Delta F_1 = RT \ln x_1 + v_1 (\delta_1 - \delta_2)^2 \phi_1^2
\]

(6.1a)

\[
\Delta F_2 = RT \ln x_2 + v_2 (\delta_1 - \delta_2)^2 \phi_2^2
\]

(6.1b)

The solubility (or immiscibility) of substance 1 into 2 is described in Equation (6.1a) and 2 into 1 in Equation (6.1b). The partial molal free energy \(\Delta F\) is a sum of the term from entropy of mixing and the heat of mixing. R is the gas constant, T is temperature, \(x_1/x_2\) are the respective mole fractions, \(v_1/v_2\) are the molal volumes, \(\phi_1/\phi_2\) are the volume fractions, and \(\delta_1/\delta_2\) are the solubility parameters of substance 1 and 2 respectively. When the solubility parameters of substance 1 and 2 equal each other \(\delta_1 = \delta_2\), there is no heat of mixing and the two components are miscible. As the difference between \(\delta_1\) and \(\delta_2\) becomes larger, the solubilities decrease.
Figure 6.2 The fluorous effect. Shown is the separation of a triphasic system of hydrocarbon, water, and perfluorocarbon solvents. Reproduced by permission of The Royal Society of Chemistry (Cametti, 2011).
resulting in phase separation. The $\delta$ of each component of a mixture can be determined using Equation 6.2 (Scott, 1948).

$$\delta = \left( \frac{\Delta E^v}{V} \right)^{1/2} \quad (6.2)$$

The solubility parameter ($\delta$) is dependent on the energy of vaporization ($\Delta E^v$) and the molal volume ($V$) of the substance. The work presented and discussed by Scott (Scott, 1948) showed that fluorocarbons have lower $\delta$ values than hydrocarbons due to lower boiling points and larger molal volumes. These properties of fluorocarbons can be attributed to differences in London Dispersion Forces between fluorocarbons and hydrocarbons (described above). These properties cause the fluorous effect and explain why fluorinated molecules preferentially dissolve/associate with other fluorinated species.

### 6.B The Vif:Elongin BC complex as a model system

A model system was needed to test the fluorous based capture system (illustrated in Figure 6.1) for HX applications. The extensive work conducted on characterizing the Vif:Elongin BC interaction presented in Chapters 4 and 5 made this system an obvious choice for the model system. Another reason why the Vif:Elongin BC system was chosen was due to the moderate affinity of Vif peptides for Elongin BC which is necessary for Elongin BC capture on a fluorous surface. The concept of using the Vif:Elongin BC for fluorous capture is illustrated in Figure 6.3. The Vif$_{138-161}$ peptide was chosen as a template to design a fluorous probe to capture Elongin BC. The extra amino acids on the C-terminal portion of this peptide, as compared to the peptides described in Chapter 4 and 5, were included to improve the solubility of the fluorous tagged version. The Vif$_{138-161}$ peptide was synthesized using solid phase peptide synthesis (see Figure 5.7 for overview of general synthesis) and two C$_6$F$_{13}$ fluorous tags were attached to the N-
Figure 6.3 The Elongin BC complex as a model system. The fluorous silica is shown as a grey sphere, Vif_{138-161} peptide in red, Elongin B in green, and Elongin C in Yellow. The Vif_{138-161}-fluorous tagged peptide was synthesized by Dr. Jason Marineau (Dana Farber Cancer Institute). Cartoon image not drawn to scale.
terminus of the Vif\textsubscript{138-161} peptide using an amine reactive, succinimide containing fluorous tag. To ensure that the fluorous tags did not interfere with Vif\textsubscript{138-161}:Elongin BC binding, a spacer was utilized to tether the peptide to the fluorous tags (see Figure 6.3). The fluorous-Vif\textsubscript{138-161} peptide was synthesized by Dr. Jason Marineau (Dana Farber Cancer Institute). The Fluorous-Vif\textsubscript{138-161} peptide served as the fluorous probe which could then be immobilized onto fluorous silica. Recombinant Elongin BC could then be captured onto the fluorous silica packed into column format.

To determine if the fluorous tags covalently attached to the Vif\textsubscript{138-161} peptide altered Elongin BC binding, the HX MS Elongin BC binding assay described in Chapter 5 was employed to test the ability of fluorous-Vif\textsubscript{138-161} to bind Elongin BC and the results shown in Figure 6.4. Elongin BC was labeled for 1 minute in D\textsubscript{2}O in the absence or presence of a two-fold molar excess of fluorous-Vif\textsubscript{138-161} and the positive control Vif peptide (P3, see Chapter 5). The conformation of Elongin C was utilized as a probe for binding as Vif association protected Elongin C from deuteration (described in Chapter 4 and 5). The results in Figure 6.4 show the Elongin C +9 charge state, that upon incubation with fluorous-Vif\textsubscript{138-161} and the positive control peptide (P3), the deuteration of Elongin C was suppressed as compared to the unbound form. These results indicate that fluorous-Vif\textsubscript{138-161} interacted with the Elongin BC complex and that addition of the fluorous tag did not appear to interfere with Elongin BC binding as detected with HX.

6.B.1 Selective capture of the Elongin BC complex with fluorous-Vif\textsubscript{138-161}

Having established that fluorous-Vif\textsubscript{138-161} bound to Elongin BC and could be used as a fluorous probe, the next step was to determine if fluorous-Vif\textsubscript{138-161} could be immobilized onto
Figure 6.4 Assaying binding between fluorous-Vif\textsubscript{138-161} and the Elongin BC complex. Deuteration results for the +9 charge state of Elongin C are shown after 1 minute in the absence of any peptide (top), incubation with the Vif P3 peptide (middle), and the fluorous-Vif\textsubscript{138-161} peptide (bottom). The unbound distribution (higher mass) is indicated by the blue dashed line while the bound distribution (lower mass) is indicated by the red dashed line.
fluorous silica packed into a column and capture Elongin BC. Before Elongin BC capture by fluorous-Vif138-161 could be attempted, the non-specific interactions of fluorous silica with Elongin BC were tested and the results are shown in Figure 6.5. Recombinant Elongin BC (500 pmols) was passed through an Alltech analytical guard column (Grace, Deerfield, IL) packed with fluorous Flash® silica gel (Fluorous technologies, Pittsburgh, PA). The spectrum in Figure 6.5A (i) shows the experimental input for Elongin BC and (ii) shows the flow-through of 500 pmols of Elongin BC through the fluorous column. There was a significant decrease in Elongin BC MS signal after flowing Elongin BC through the fluorous column which suggested that Elongin BC interacted non-specifically with fluorous silica gel. Protein adsorption onto silica gel has been previously documented due to hydrogen bonding and non-specific interactions between proteins and the silanol groups of silica gel (Tatsuya, 2006; Suh, 2004; Tsukagoshi, 2007). These reports also suggested a link between silica gel pore size and levels of protein adsorption. Other published applications utilizing fluorous protein capture on different supports (i.e. solid silica glass slides) have shown that the fluorous system to have low levels of non-specific binding (Vegas, 2007; Vega, 2010; Nicholson, 2007). The results presented in this Chapter and those reported in the literature indicate that the non-specific binding of Elongin BC was likely occurring with the silica gel and not the fluorous surface itself. To circumvent the non-specific binding of Elongin BC to the silica, the fluorous column was passivated with BSA and washed prior to incubation with Elongin BC. The results shown in Figure 6.5B illustrates the Elongin BC input (i) and column flow-through (ii) of Elongin BC through a passivated fluorous column. The MS signal for Elongin BC before and after the fluorous column were similar suggesting that BSA passivation eliminated the non-specific interactions between Elongin BC and the fluorous silica.
Figure 6.5 Passivation of fluorous silica. ESI mass spectra of 500 pmols of Elongin BC before (i) and after (ii) flowing through a non-passivated fluorous column (A) and BSA passivated fluorous column (B). The charge envelope for Elongin B is indicated by the green dots, and Elongin C by yellow dots.
The ability of fluorous-Vif$_{138-161}$ to bind to fluorous silica and capture Elongin BC was then tested by loading 125 nmols of modified peptide through the fluorous column; washing extensively, and then flowing Elongin BC through the column. A non-fluorous tagged Vif peptide was used as a negative control. In principle, if the fluorous-Vif$_{138-161}$ bound to the fluorous silica and Elongin BC was passed through the column, no Elongin BC should be present in the column flow through as Elongin BC should be specifically retained on the surface. The non-fluorous tagged peptide should not associate with the fluorous silica and flow directly through the column. No retention of Elongin BC should be observed upon incubation with fluorous silica incubated with the non-fluorous tagged peptide. The results are shown in Figure 6.6 for Vif non-fluorous tagged peptide (A) and fluorous-Vif$_{138-161}$ (B). The Elongin BC experimental input before flowing through the fluorous column is shown in (i) and the column flow-through in (ii). The results in (A) show that the MS signals for Elongin BC before and after incubation with the fluorous column were similar. The results indicate that Elongin BC was not captured on the fluorous surface. The results in (B) for fluorous-Vif$_{138-161}$ show that Elongin BC was not present in the column flow-through and was instead captured by fluorous-Vif$_{138-161}$ that was bound to the fluorous surface. A denaturing HX quench solution (see material and methods) was passed through the column to denature and elute bound Elongin BC and the results in (iii) show that this step was successful at eluting Elongin BC. Note, a small amount of the fluorous-Vif$_{138-161}$ was present in the samples shown in (ii) and (iii) indicating that the initial washing of the fluorous column after loading fluorous-Vif$_{138-161}$ did not completely remove all of the unbound fluorous-Vif$_{138-161}$ peptide. Washing of the fluorous column with 1000 column volumes of 20% methanol followed by 20 column volumes of wash buffer resolved this issue. This is
Figure 6.6 Selective capture of recombinant Elongin BC with fluorous Vif$_{138-161}$. ESI mass spectra of 500 pmols of Elongin BC before (i) and after (ii) flowing through passivated fluorous columns loaded with a non-fluorous Vif peptide (A) or fluorous-Vif$_{138-161}$ (B). The quenched sample from the fluorous-Vif$_{138-161}$ loaded column is shown in (iii). The charge envelope for Elongin B is indicated by the green dots, Elongin C by yellow dots, and fluorous-Vif$_{138-161}$ by blue asterisks.
illustrated by the lack of the fluorous-Vif$_{138-161}$ signal in the sample shown in Figure 6.7B which is a replicate of the sample shown in Figure 6.6B using an extensively (see above for wash).

### 6.B.2 Hydrogen exchange of Elongin BC on fluorous silica

The results in Section 6.B.1 illustrate that the Elongin BC complex could be captured by the fluorous-Vif$_{138-161}$ peptide immobilized onto fluorous silica packed into a column format. The next step was to see if HX MS analysis of Elongin BC could be conducted using the fluorous capture system and if deuteration results were similar to those that were observed using a solution based HX MS protocol. For solution based HX MS experiments, the Elongin BC complex was incubated with a 2.5-fold molar excess of fluorous-Vif$_{138-161}$ in solution and samples were labeled and analyzed as described in Chapter 2 (see also material and methods). A representative mass spectrum from the solution based HX MS analysis of Elongin BC is shown in Figure 6.7A. One limitation of conducting binding studies with solution based HX MS is that the excess ligand present (fluorous-Vif$_{138-161}$) in solution that is required to ensure maximal protein binding (Elongin BC) can interfere with the MS analysis of the protein of interest. Figure 6.7A shows that the mass spectrum of the fluorous-Vif$_{138-161}$:Elongin BC mixture is dominated by the signal of fluorous-Vif$_{138-161}$. This problem is eliminated when using the fluorous capture system as the Vif peptide was retained on the fluorous surface as illustrated in Figure 6.7B. For labeling experiments using the scheme described in Figure 6.1, the Elongin BC complex was captured in the fluorous column and labeling initiated by rapidly flowing 1 mL of D$_2$O through the column and then continuously flowing D$_2$O through the column for the desired labeling time. The earliest labeling time conducted in these experiments was 2 minutes. Manually controlling the D$_2$O flow into the fluorous column was challenging and is one current limitation to this
Figure 6.7 Advantage of fluorous capture vs. solution exchange. ESI mass spectra of 500 pmols of Elongin BC from solution based (A) and fluorous based (B) HX experiments. The charge envelope for Elongin B is indicated by the green dots, Elongin C by yellow dots, and fluorous-Vif_{138-161} by blue asterisks.
methodology as short labeling times (< 1 minute) are difficult to conduct. The quench step was conducted post labeling by rapidly flowing quench solution through the column. This rapid buffer exchange served two purposes: (1) quench the amide HX reaction, and (2) denature and elute the Elongin BC complex from the fluorous surface. Eluent from the column upon quenching was manually collected in a microcentrifuge tube and then mass analyzed. A representative mass spectrum from the fluorous based HX MS analysis of Elongin BC is shown in Figure 6.7B and shows the advantage in MS analysis of immobilizing the fluorous-Vif138-161 peptide onto the fluorous silica. The summary of HX MS analysis for Elongin BC in solution and using the fluorous capture system is shown in Figure 6.8. Deuteration monitored from the +9 charge state of Elongin C is shown in Figure 6.8A and the +11 charge state for Elongin B (B) using the fluorous capture system (i) and in solution (ii). The results shown illustrate that the fluorous system can be used to monitor deuteration in both Elongins and that the deuterium uptake curves in (iii) show similar deuteration for both Elongins when HX is conducted on the fluorous surface or in solution. The results shown indicate on the intact level that immobilization onto a fluorous surface did not alter Elongin BC conformation and that the fluorous based platform can be utilized for HX MS applications.

6.B.3 Selective capture of Elongin BC from an E. coli lysate

The results presented in the previous sections show the utility of a fluorous capture system for HX MS applications. A major limitation of conducting HX MS using conventional solution based techniques is that the protein system of interest needs to be isolated prior to biophysical analysis as illustrated in Figure 6.9A. Protein expression and purification using conventional techniques can be challenging and include multiple rounds of purification to isolate
Figure 6.8 HX MS analysis of Elongin BC on fluorous silica. Deuteration of the +9 charge state of Elongin C (A) and +11 charge state of Elongin B (B) using the fluorous capture system (i) compared to solution based experiments (ii). Deuterium uptake curves for Elongin B and C are shown in (iii). Deuteration using the fluorous system is highlighted in red and solution based results in black.
pure recombinant protein. Major pitfalls when using the scheme in Figure 6.9A are that protein purification can be time consuming and large multi-protein complexes can be difficult to isolate. The workflow in Figure 6.9B illustrates the use of the fluorous capture system to isolate a protein target for biophysical analysis. A major advantage of using the fluorous based system is that the target protein can be selectively isolated from a complex mixture such as an *E. coli* or mammalian cell lysate in fewer steps than the workflow shown in Figure 6.9A. Protein targets captured with this method can then be subjected to HX MS and other biological analysis.

To test the ability of the fluorous based system to selectively capture a target protein from a complex mixture, an *E. coli* lysate was produced from cells expressing Elongin BC [see Chapter 4 and (Marcisbin, 2010) for Elongin BC preparation] and a mass spectrum taken as shown in Figure 6.10A. The peaks in Figure 6.10A correspond to the various soluble *E. coli* proteins and Elongin BC. The lysate was then flowed through a fluorous-Vif_{138-161} loaded fluorous column (as described above) and any unbound *E. coli* proteins removed by washing the column with 50 column volumes of wash buffer (see material and methods). After washing the column, 250 uL of wash buffer was then flowed through the column; collected, and mass analyzed. The mass spectrum in Figure 6.10B shows that extensive washing removed all unbound proteins from the fluorous column. To elute captured Elongin BC, quench buffer was flowed through the fluorous column and the eluent collected and mass analyzed. The spectrum in Figure 6.10C shows the charge envelopes for both Elongins and significantly fewer contaminant peaks than the spectrum in Figure 6.10A illustrating that Elongin BC was selectively captured from the *E. coli* lysate. The results in Figure 6.10 demonstrate that the fluorous capture system is capable of selectively isolating a target protein from a complex mixture which can then be analyzed with different assays including HX MS.
Figure 6.9 Conventional protein purification vs. fluorous capture.
(A) Illustrated are some common steps when attempting to isolate a recombinant protein system for biophysical analysis. Purification needs are protein dependent and the steps are shown to illustrate the complexities in purification. (B) Workflow for capturing a target protein using the fluorous based approach.
Figure 6.10 Capture of the Elongin BC complex from an *E. coli* lysate. (A) ESI mass spectrum of an *E. coli* lysate containing Elongin BC. The lysate shown in Panel A was passed through a fluorous-Vif$_{138-161}$-loaded fluorous column; the column washed extensively, and bound proteins eluted by quenching. The ESI mass spectra for the washing and elution steps are shown in (B) and (C) respectively. The charge envelope for Elongin B is indicated by green dots and Elongin C by yellow dots.
6.C Design of fluorous flow system for HX MS applications

The work presented in Sections 6.A and B illustrate the potential use and demonstrates the feasibility of fluorous chemistry for HX MS applications. The utility of the fluorous capture concept would be greatly improved if an automated system were developed that would reduce manual sample handling. The valve systems illustrated in Figure 6.11 could potentially be used to automate fluorous capture and HX MS reactions. The setup in Figure 6.11A would be applicable to intact analysis of protein and protein complexes. A 6-way switching valve would control which samples and buffers would flow into the fluorous column. The flow of each component could be controlled by syringe pumps or an HPLC. Protein samples that are captured and labeled using the fluorous column would then be directed post quenching through a Rheodyne 7000 switching valve where desalting and elution into the mass spectrometer would occur.

Ultimately, the goal for the HX MS analysis of proteins using the fluorous capture system would to be able to conduct conformational studies on both the intact and peptide levels. The setup illustrated in Figure 6.11B could be utilized for peptide HX MS analysis of proteins and protein complexes. The workflow for the peptide system would be similar for capture and labeling as that illustrated in Figure 6.11A; however, post quenching protein samples would be digested using a pepsin column. Peptic peptides would then be trapped, desalted, separated and eluted before MS analysis.

To determine if a valve system could be utilized with a fluorous column, The valve system shown in Panel A was constructed and tested to see if Elongin BC could be captured and eluted. 500 pmols of Elongin BC were flowed through a fluorous-Vif138-161 loaded fluorous column (green); the column washed extensively, and the remaining bound protein eluted by
Figure 6.11 Fluorous flow system. (A) Design of valve setup for intact protein analysis using the fluorous capture system. (B) Design of value setup for peptide analysis using the fluorous capture system. (C) ESI mass spectrum of 500 pmols of Elongin BC captured (process illustrated in in Figure 6.6B) using the valve setup shown in Panel A. The charge envelope for Elongin B is indicated by green dots and Elongin C by yellow dots.
quenching (process illustrated in Figure 6.6B). The protein loading, column washing, and elution steps were conducted using a syringe pump. The results for the quenched sample are shown in Figure 6.11C and illustrate that Elongin BC was successfully captured and then eluted from the fluorous column using the valve system shown in Panel A.

6.D Summary

The work presented in this Chapter illustrates that fluorous chemistry can be utilized in HX MS applications. The fluorous system has the possibility to alleviate several issues with conventional in-solution labeling including the need to fully purify a desired protein system, manual labeling, and sample dilution into D$_2$O. The work described shows that fluorous tags can be successfully attached to peptides and immobilized on fluorous silica. The immobilized fluorous-Vif$_{138-161}$ in this work was successful at capturing Elongin BC from a purified solution and from an E. coli lysate. This concept could be utilized for any protein:ligand interaction including protein:protein, protein:small molecule, or protein:oligonucleotide interactions. Several limitations of the fluorous technique include: (1) that proper passivation steps need be taken in order to eliminate non-specific interactions with the fluorous silica, and (2) that the protein:ligand interaction has to be of moderate to high affinity ($\leq 1$ µM) to retain the desired protein on the surface for HX analysis. Despite the limitations, HX MS analysis of recombinant Elongin BC using the fluorous capture system illustrates that HX on a surface support is possible. One important concern when conducting HX on a surface is that the surface does not alter protein conformation. The similar deuteration results of Elongin B and C in solution as compared to the fluorous column shows that fluorous media did not alter exchange in the context of Elongin BC and likely does not in other protein systems. The design and building of the value
setup shown in Figure 6.11 demonstrates that a flow system can be constructed which has the potential for automation alleviating the issue of manual labeling.

6.E Materials and methods

6.E.1 Mass analysis of fluorous samples

Samples were injected into a Shimadzu SCL-10A VP HPLC flowing water containing 0.05% formic acid, pH 2.6 at 50 μL/min coupled to a Waters LCT premier mass spectrometer with a standard electrospray interface. Protein samples were trapped and desalted using an Alltech analytical in-line guard column, packed with POROS 20-R2 reversed-phase media (PerSeptive Biosystems) and eluted directly into the mass spectrometer with a gradient of 15-98% acetonitrile (containing 0.05% formic acid, pH 2.6) in 5 minutes. For HX MS experiments with Elongin BC, the injector, column and all associated tubing were kept at 0 °C to minimize back exchange. Mass determination and deuterium content were determined as described in Chapter 2.

6.E.2 Synthesis of fluorous-Vif138-161

Synthesis of the fluorous-Vif138-161 peptide was conducted by Dr. Jason Marineau (DFCI). Briefly, the fluorous Vif peptide (FluorFmoc-PEG2-GHNKVSLQYLALAALITPKIKK-NH2) was synthesized on 100 μmol scale using standard 9-fluorenlymethoxycarbonyl (Fmoc) peptide chemistry on NovaPEG Rink amide resin using a CEM Liberty 9008005 microwave peptide synthesizer. Subsequent transformations were conducted using manual peptide synthesis techniques. After removal of the N-terminal Fmoc group, the resin was suspended in DMF and coupled with Fmoc-NH-
and DIPEA (5 equiv.). After subsequent Fmoc deprotection (20% Piperidine/DMF), the resin was suspended in dichloromethane and fluorous tagged using N-[2,7-Bis(1H,1H,2H,2H-perfluorooctyl)-9-fluorenymethoxycarbonyloxy] succinimide (Fluorous Technologies Incorporated F026005, 2 equiv.) and DIPEA (5 equiv.). The peptide was then cleaved from the resin using 95% TFA/2.5% TIS/2.5% H2O and precipitated in Et2O to afford the crude, fluorous tagged, C-terminal amidated peptide. The peptide was purified on a Varian ProStar HPLC using a Dynamax 21.4 X 250 mm 300 Å C18 column eluted with water-acetonitrile containing 0.1% TFA as the mobile phase and UV monitoring at 220 nm. The fractions were collected and the solvent removed on a lyophilizer to produce 126 mg of the desired peptide as a dry powder.

6.E.3 Protein expression and purification

Preparation of recombinant Elongin BC complex and the Vif135-158 peptide was conducted as described in Chapter 4. For the preparation of the E. coli lysate utilized in the experiments described in Section 6.B.3, a 2 g pellet of E. coli cells was re-suspended in lysis buffer containing 20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol, pH 7.0, and supplemented with PMSF and lysozyme. Re-suspended cells were lysed by sonication and the soluble protein fraction isolated by centrifugation at 30,000 rpm for 40 minutes. The soluble protein fraction (supernatant) was separated from the insoluble fraction and used as described below.

6.E.4 Preparation of fluorous flash® packed column

An Alltech® (Grace, Deerfield, IL) analytical in-line guard column (3 mm x 22 mm id) was dry packed with ~ 7 mg of FluoroFlash® (40 µm, 60 Å pore diameter, 0.7 pore volume)
(Fluorous Tech Inc, Pittsburg, PA) silica gel. The packed column void volume (value used to determine column volume equivalents) was calculated using Equation 6.3 and determined to be 108 µL

\[ V_{\text{void}} = \left( \frac{\pi d^2 V_{\text{pore}} L}{4000} \right) \]  

(6.3)

The void volume \( V_{\text{void}} \) was calculated using the diameter \( d \), length of the column \( L \), and pore volume \( V_{\text{pore}} \). The packed fluorous column was washed with 20 column volumes of DMSO, distilled water, and wash buffer (20 mM HEPES, 150 mM NaCl, 1 mM DTT, 10% glycerol at pH 7.0). All wash steps and column incubations were conducted by hand using a 250 µL Hamilton syringe (Reno, NV) unless otherwise stated. The fluorous silica was passivated by flowing 1 mL of 1.46 mg/mL BSA (Biorad, Hercules, CA) through the packed column. Unbound BSA was removed by washing with 20 column volumes of wash buffer. Non-specific binding of Elongin BC was tested by flowing 500 pmols of protein (100 µL of 5 µM) through the fluorous column pre and post BSA passivation using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. The flow-through and a 2 column volume wash was collected and mass analyzed as described above.

### 6.E.5 Immobilization of fluorous-Vif\textsubscript{138-161} and capture of Elongin BC

All fluorous immobilizations and captures were conducted at 25 °C. To immobilize fluorous-Vif\textsubscript{138-161} onto the fluorous silica, 125 nmols of the peptide (250 µL of 500 µM) was passed through the fluorous column using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. To test the specific binding of fluorous-Vif\textsubscript{138-161} to fluorous silica, the non-fluorous tagged Vif\textsubscript{135-158} peptide was incubated with a packed fluorous column and washed.
as described for fluorous-Vif\textsubscript{138-161}. Unbound fluorous-Vif\textsubscript{138-161} was removed by washing with 800-1000 column volumes of 20% methanol using a Shimadzu (Canby, OR) SCL-10A VP HPLC, followed by 20 column volumes of wash buffer.

To capture purified Elongin BC or from an \textit{E. coli} lysate, 500 pmols of purified protein (100 µL of 5 µM) or 100 µL’s of lysate were flowed through a fluorous-Vif\textsubscript{138-161} loaded fluorous column using a Harvard Apparatus (Holliston, MA) syringe pump flowing at 15 µL/min. The fluorous column was then washed with 50 column volumes of wash buffer. Captured Elongin BC was eluted from the fluorous column by rapidly flowing 15 column volumes of quench buffer (0.8% formic acid, 0.8 M guanidine hydrochloride, pH 2.2) and mass analyzed as described above.

6.E.5 HX reactions

Solution based HX reactions were conducted by incubating Elongin BC with a 2.5 molar excess of the fluorous-Vif\textsubscript{138-161} at 25 °C for 30 minutes. Equilibrated samples were then labeled by a 10-fold dilution in D\textsubscript{2}O buffer (20 mM, HEPES, 1 mM DTT, 150 Mm NaCl). After the desired labeling times, samples were quenched to pH 2.0 by addition of a 4:1 ratio (v/v) of quench buffer (0.8 M GdnHCl, 0.8% FA, pH 2.0) to each protein sample. The pH of the quenched samples had to be below the typical pH 2.6 value as no Elongin C elution was observed using the fluorous system at pH 2.6. For fluorous based HX experiments, the Elongin BC complex was captured onto a fluorous-Vif\textsubscript{138-161} loaded column and labeling was initiated by rapid manual flushing (~ 3 mL/min) of the column with 250 µL of D\textsubscript{2}O buffer (see above). After initial flushing of the fluorous column with D\textsubscript{2}O buffer, a continuous flow of D\textsubscript{2}O buffer was maintained through the column using a Harvard Apparatus (Holliston, MA) syringe pump.
flowing at 15 µL/min. The actual reported labeling times in the work presented accounted for the initial D₂O buffer flushing and flow using the syringe pump. HX reactions were then quenched (see above for quench buffer) by disconnecting the flow of D₂O buffer and then rapidly flowing (~ 3 mL/min) 15 column volumes of quench buffer through the column. Column eluent from the quenching step was collected in 1.6 µL microcentrifuge tubes and then mass analyzed as described above.

6.F References


CHAPTER 7
Perspectives and Future Directions

Several important observations can be made from the research presented. HIV-1 Vif produced recombinantly retained a biochemically relevant conformation in the context of SFK and Elongin BC binding when isolated under denaturing conditions, and renatured. Renatured Vif was amenable to conformational analysis using HX MS. Conformation results from HX revealed that the N-terminal region that is responsible for APOBEC3 association likely contained the majority of structural elements while the C-terminal portion was dynamic and solvent exposed in solution. Another highlight of this work was the ability to probe the interaction between full length Vif and the E3 ligase components Elongin BC for the first time. Information obtained from the HX MS analyses of the Vif:Elongin BC complex has led to the development of unique stapled peptide inhibitors towards the Vif:Elongin BC interaction. The results presented for HIV-1 Vif are some of the most detailed conformational information reported for the full length protein and provide the foundation to probe the interactions between Vif and host cell proteins such as APOBEC3G.

In the future, several aspects of Vif can be further investigated to advance the study of Vif biology and the field of virology.

7.A Conformational investigation of the Vif:APOBEC3G complex and host cell partners

Other than Vif, there is also a lack of conformational information on the APOBEC3 enzymes and the E3 ligase that Vif recruits. There is no three dimensional structure for a full length APOBEC3 protein to date, as only the C-terminal domain of APOBEC3G was amenable
to NMR and X-ray analyses (Chen, 2008; Furukawa, 2008; Furukawa, 2009; Harjes, 2009; Shandilya, 2010). The published structures only contain information for residues 191-384 of APOBEC3G and do not elucidate the conformation of the N-terminal region required for Vif binding (residues 54-124, and D128) (Chiu, 2009). A full length construct of APOBEC3G could be created; expressed in insect cells, and purified. Such a construct would allow for the HX MS conformational investigation of APOBEC3G. Such studies would elucidate any changes that might result from Vif:APOBEC3G complexation.

Cullin 5 is another host cell protein that interacts with Vif and lacks structural data. The Cullin 5 protein acts as the main scaffolding component of the E3 ligase complex responsible for APOBEC3G ubiquitylation. The exact mechanism of how Vif associates with Cullin 5 is unknown as it was once hypothesized that the Vif:Cullin 5 association was solely mediated through the Vif zinc finger (Yu, 2004). A recent report has shown that Vif from the feline immunodeficiency virus, which does not contain a zinc binding domain, is capable of recruiting the Cullin 5 ligase (Wang, 2011). A full length construct of Cullin 5 could be created; expressed in E.coli, and purified. Recombinant Cullin 5 would allow for the HX MS conformational investigation of the Vif:Cullin 5 association. Such results could shed light on the mechanisms by which Vif recruits Cullin 5.

7.B Conformational analysis of Vif proteins from different HIV strains

Vif proteins from different HIV strains and immunodeficiency viruses (i.e. feline immunodeficiency virus, and simian immunodeficiency virus), differ in their potency with which they are able to counteract the APOBEC3 enzymes (Binka, 2011; Iwabu, 2010; Ribeiro, 2005). These proteins vastly differ in their primary structure as sequence alignment of known lentiviral
Vif proteins show less than 30% sequence identity (Oberste, 2000; Shacklett, 1994; LaRue, 2010) and it is unknown how they vary structurally. The work presented in this dissertation illustrates the ability of HX MS to probe Vif conformation and could be used to study different Vif proteins. Such an analysis might elucidate how different Vif proteins with low sequence identity are still able to direct the APOBEC3 family of enzymes for ubiquitylation.

7.C Further development of Vif based inhibitors

The work described in Chapter 5 illustrated the potential to use Vif peptides to inhibit HIV-1 replication. The work presented provides the foundation for the further development of Vif:Elongin BC stapled peptide inhibitors. Various lengths of Vif sequence could be screened, as well as the different stapling systems (i.e., i+3, i+7), to produce an enhanced version of the peptide described in Chapter 5. It would be interesting to screen these peptides for anti-HIV activity in different non-permissive cell lines (cells that express APOBEC3G, Vif is required for HIV infection) and then screened in animal models to see if the peptides exhibit antiviral activity on the organism level. Similar efficacy studies were conducted for stapled peptide inhibitors of NOTCH signaling in a mouse model (Moellering, 2009).

Several interesting observations can be made from the work in Chapter 3. Recombinant HIV-1 Vif interacted with, and activated, members of the Src family of tyrosine kinases (SFK). The exact role of the Vif:SFK interaction in the context of HIV biology is not fully understood. Vif dependent activation of the kinases might support a meaningful role in HIV replication and the assays described in Chapter 3 could be utilized to screen libraries of small molecule inhibitors towards Vif dependent kinase activation, as was previously reported for another HIV-1
accessory protein (Emert-Sedlak, 2009). The potential inhibitors listed above might provide additional therapies against the human immunodeficiency virus.

7.D References


APPENDIX I

Publication


Special focus issue: Structural and Molecular Biology of HIV
On the Solution Conformation and Dynamics of the HIV-1 Viral Infectivity Factor

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Human immunodeficiency virus-1 (HIV-1) has evolved a cunning mechanism to circumvent the antiviral activity of the APOBEC3 family of host cell enzymes. HIV-1 Vif [viral (also called virion) infectivity factor], one of several HIV accessory proteins, targets APOBEC3 proteins for proteasomal degradation and downregulates their expression at the mRNA level. Despite the importance of Vif for HIV-1 infection, there is little conformational data on Vif alone or in complex with other cellular factors due to incompatibilities with many structural techniques and difficulties in producing suitable quantities of the protein for biophysical analysis. As an alternative, we have turned to hydrogen exchange mass spectrometry (HX MS), a conformational analysis method that is well suited for proteins that are difficult to study using X-ray crystallography and/or NMR. HX MS was used to probe the solution conformation of recombinant full-length HIV-1 Vif. Vif specifically interacted with the previously identified binding partner Hck and was able to cause kinase activation, suggesting that the Vif studied by HX MS retained a biochemically competent conformation relevant to Hck interaction. HX MS analysis of Vif alone revealed low deuteration levels in the N-terminal portion, indicating that this region contained structured or otherwise protected elements. In contrast, high deuteration levels in the C-terminal portion of Vif indicated that this region was likely unstructured in the absence of cellular interacting proteins. Several regions within Vif displayed conformational heterogeneity in solution, including the APOBEC3G/F binding site and the HCCH zinc finger. Taken together, these HX MS results provide new insights into the solution conformation of Vif.

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Introduction

Human immunodeficiency virus (HIV) belongs to the Retroviridae family of viruses and differs from primate retroviruses in that HIV requires the expression of additional proteins besides Gag, Pol, and Env for efficient and productive viral infection (see Frankel and Young¹ and Seelamgari et al.² for
reviews). These additional proteins can be classified into regulatory and accessory proteins. The regulatory proteins consist of Tat and Rev, and are responsible for viral gene regulation, while the accessory proteins consist of Vif, Vpr, Nef, and Vpu. The accessory proteins play a pivotal role in viral pathogenesis by acting as versatile adaptors bridging viral and cellular pathways necessary for infection and immune evasion.

HIV-1 Vif [viral (also called virion) infectivity factor] has been shown to be essential for viral pathogenesis. Vif is a 22.5-kDa highly basic protein that interacts with an array of both cellular proteins and DNA/RNA. In the absence of Vif, members of the APOBEC3 family of cytidine deaminases, including APOBEC3F/G, are packaged into HIV virions. Upon virus entry into subsequent cells, APOBEC3F/G inhibits viral replication through mechanisms that are both dependent on and independent of its deaminase activity. In the deaminase-independent mechanism, APOBEC3 inhibits reverse transcription. The deaminase-dependent mechanism involves deamination of cytidines to uridines in the (+) strand of the viral DNA, causing a crippling G-to-A hypermutation that renders subsequent viral infection nonproductive. HIV-1 Vif, however, circumvents the antiviral activities of the APOBEC3 proteins by several mechanisms, including: (1) inhibition of APOBEC3 mRNA translation, (2) promotion of the formation of high-molecular-weight APOBEC3 complexes, and (3) targeted proteasomal degradation wherein Vif links the APOBEC3 enzymes to components of the Elongin BC–Cullin 5 ubiquitin ligase complex.

Vif lacks known intrinsic enzymatic activity and functions instead by interacting directly with APOBEC3 proteins, the ubiquitylation machinery, and other cellular factors. Figure 1 summarizes the structural regions of Vif and the known functions ascribed to each region. The N-terminal portion of Vif (Fig. 1, blue) contains several APOBEC3F/G binding motifs and is also essential for DNA/RNA interaction. There is a novel HCCH zinc finger in the central region of Vif (Fig. 1, red) that is responsible for the interaction with the E3 ligase component Cullin 5. The C-terminal portion of Vif contains multiple motifs, including a novel viral SOCS (suppressor of cytokine signaling) box (Fig. 1, black), which recruits the E3 ligase scaffold protein complex Elongin BC. The PPLP domain (Fig. 1, orange) is just C-terminal to the viral SOCS box and has been implicated in several functions, including Vif multimerization and interaction with both the tyrosine kinase Hck and APOBEC3G. The extreme C-terminal domain of Vif is required for association with gag, NCp7, and the cellular membrane.

The structural features of Vif that enable interactions with such a diverse group of host cell factors are not well defined. No three-dimensional structure of full-length Vif has been reported to date, although lower-resolution structural techniques and algorithms such as PONDR VL-XT indicate that
Vif contains some structure spread throughout the protein\textsuperscript{43} (Fig. 1, bottom). Segments in the N-terminus and the last 40 residues in the C-terminus are predicted to be unstructured. These unstructured regions are likely to be conformationally flexible, thus enabling Vif to adopt the correct shape required for interaction with APOBEC3F/G and other host cell factors. Other regions are predicted to contain some structural elements. To characterize the various regions of Vif (those predicted by algorithms to contain structure and those that are essential for interacting with other proteins), we turned to an alternative biophysical technique for conformational characterization, hydrogen exchange mass spectrometry (HX MS).

HX MS is a technique that is advantageous over other biophysical techniques such as X-ray crystallography and NMR because it is compatible with proteins such as Vif, which are aggregation prone and difficult to obtain in suitable quantities for analysis.\textsuperscript{44} Very little material is required (as little as 500–1000 pmol for an entire experiment), and the concentration of the material can be quite low (as low as 0.1 \textmu M). Thus, for proteins that are difficult to purify or otherwise hard to obtain in suitable quantities for characterization by other more classical tools, some information can still be obtained. HX MS cannot be used to solve the structure of a protein, but it can be used to glean conformational information, to detect conformational changes, and to differentiate protein sub-populations in solution.\textsuperscript{44,45} We recently used HX MS to probe the binding between full-length recombinant Vif and the Elongin BC complex.\textsuperscript{46}

In the current study, HX MS was used to probe Vif conformation in detail. HX MS binding studies and tyrosine kinase assays revealed that recombinant full-length Vif specifically interacted with both the isolated Hck Src homology 3 (SH3) domain and the near-full-length Hck. Interaction with Vif induced activation of Hck, a member of the Src kinase family that is prone to activation by SH3 binding proteins.\textsuperscript{47} Interaction with Vif was also found to activate the Elongin BC complex.\textsuperscript{46} Hck SH3 domain binding is a known regulatory component of Hck and is also targeted by another HIV-1 accessory protein, Nef.\textsuperscript{51,52} Nef/Hck interaction between Vif and the tyrosine kinase Hck. We have previously shown that Vif prepared in the same manner bound efficiently to the Elongin BC complex.\textsuperscript{46} The refolded recombinant Vif was highly amenable to analysis by electrospray ionization mass spectrometry, as shown in Fig. 2a. To determine if the refolded material had any structure, we analyzed refolded Vif with circular dichroism (CD), as shown in Fig. 2b. Refolded Vif was also placed in 8 M guanidine hydrochloride, and a CD spectrum was obtained. Subsequent analysis of the CD spectra with the software program CDPro\textsuperscript{50} (Fig. 2c) suggested that much of Vif was unstructured, with some secondary structure present as \(\beta\)-sheet. In guanidine hydrochloride, the secondary structure distribution relative to refolded Vif shifted to less \(\alpha\)-helical content, although \(\beta\)-sheet elements were largely retained. Our CD results are consistent with previous work in which the majority of secondary structural elements in recombinant refolded Vif were \(\beta\)-sheet.\textsuperscript{49}

To verify that recombinant full-length Vif, expressed and purified according to the Yang protocol,\textsuperscript{48} had a biochemically competent conformation relevant to interaction with and modulation of the Src family tyrosine kinases, we monitored the interaction between Vif and the tyrosine kinase Hck. We have previously shown that Vif prepared in the same manner bound efficiently to the Elongin BC complex.\textsuperscript{46} Hck SH3 domain binding is a known biochemical function of Vif, and this binding alters Hck kinase activity.\textsuperscript{39,40} The SH3 domain is a central regulatory component of Hck and is also targeted by another HIV-1 accessory protein, Nef.\textsuperscript{51,52} Nef/Hck SH3 interaction has been extensively studied using HX MS.\textsuperscript{47,52–54} In those prior studies, it was shown that the Hck SH3 domain itself undergoes slow cooperative unfolding in solution, which can be monitored with HX MS due to the appearance of two mass distributions corresponding to folded and

Results and Discussion

Recombinant Vif retains a biochemically competent conformation amenable to biophysical analysis

Recombinant Vif expressed in \textit{Escherichia coli} without the aid of solubility enhancement tags (such as glutathione S-transferase) localizes to insoluble inclusion bodies.\textsuperscript{48} Vif in inclusion bodies can be isolated under denaturing conditions, purified with Ni-NTA affinity chromatography, and then refolded by subsequent dialysis with buffers containing decreasing concentrations of denaturant.\textsuperscript{29,46,48,49} Hck SH3 domain binding is a known regulatory component of Hck and is also targeted by another HIV-1 accessory protein, Nef.\textsuperscript{51,52} Nef/Hck SH3 interaction has been extensively studied using HX MS.\textsuperscript{47,52–54} In those prior studies, it was shown that the Hck SH3 domain itself undergoes slow cooperative unfolding in solution, which can be monitored with HX MS due to the appearance of two mass distributions corresponding to folded and
unfolded species. Protein/peptide binding slows the rate of SH3 domain unfolding; therefore, by monitoring the rate of unfolding, we can detect and quantify protein/peptide binding to SH3. To interrogate the ability of recombinant Vif to interact with Hck SH3, we monitored the rate of SH3 unfolding by HX MS.

Figure 3 shows the results of the Vif/Hck SH3 binding assay. Hck SH3 was labeled with $^2$H$_2$O in the absence or in the presence of Vif. A wide $m/z$ distribution characteristic of protein unfolding in the mass spectra (Fig. 3a) occurred after approximately 30 min of deuterium labeling in the absence of Vif, but at a much longer time (~2 h) in the presence of Vif (note that when Nef binds to Hck SH3, almost all unfolding is abolished at very long incubation times; see Engen et al., 47,54). This shift in the unfolding half-life indicates that Vif bound to and altered the solution dynamics of Hck SH3. Changes in Hck SH3 unfolding can be quantified by calculating a slow-down factor, as shown in Fig. 3b. The presence of Vif slowed wild-type Hck SH3 unfolding by a factor of 4.1, a value that is indicative of relatively strong binding. To confirm that this Vif/SH3 association was specific, we compared the wild-type Hck SH3/Vif interaction to interaction with a nonbinding Hck SH3 mutant. Mutation of W93 to alanine in Hck SH3 abolishes the Vif/SH3 interaction, as well as other Hck SH3 binding events with other proteins. Slow-down factor analysis for Hck SH3 W93A alone and in the presence of Vif revealed that Vif had little effect on the unfolding of W93A and thus was no longer capable of binding to Hck SH3 W93A in vitro. These results confirm that suppression of Hck SH3 unfolding was a direct result of specific binding between recombinant refolded Vif and wild-type Hck SH3.

The ability of Vif to associate with the Hck SH3 domain in vitro confirmed that the protein had refolded into a biochemically competent conformation relevant to Hck interaction. This observation raised the question of whether this recombinant Vif could also activate near-full-length Hck through SH3 domain displacement, as shown previously for Nef. As shown in Fig. 3c, purified Vif was a remarkably efficient activator of Hck in vitro, with enhanced kinase activity observed at a substoichiometric Vif/kinase molar ratio. Similar results were also obtained for several other Src family tyrosine kinases expressed in HIV target cells, including Lyn and Lck (see Supplementary Material, Fig. S1).

To determine whether Vif could activate Hck in a cell-based system, we employed the yeast growth suppression assay previously used to probe Src family kinase activation by HIV-1 Nef. Yeast cells do not express orthologs of mammalian tyrosine kinases, and ectopic expression of Src family members perturbs normal cellular signaling, resulting in growth suppression. We reasoned that if Vif was coexpressed with a downregulated form of Hck (Hck YEEI) in yeast, growth would only be suppressed if the kinase became activated by Vif. To test this idea, we transformed the yeast cultures with Vif and downregulated Hck either alone or in combination and spotted them on agar plates in the presence of galactose to induce protein expression. As shown in Fig. 3d, expression of either Vif or downregulated Hck alone did not alter yeast growth. In contrast, coexpression of Vif and Hck
resulted in strong growth suppression, equivalent to that observed with wild-type active Hck. Suppression of yeast growth correlated with enhanced phosphorylation of yeast cell proteins, and similar results were obtained for the Src family tyrosine kinases Lck and Lyn (see Supplementary Material, Fig. S2). These data indicate that Vif associated with Hck in yeast cells and stimulated kinase activity, consistent with in vitro kinase assays. Taken together, the in vitro and in vivo data support previous biochemical reports that Vif interacts with Hck and alters the kinase activity of Hck.

Taken together, the in vitro HX MS Hck SH3 domain binding experiments and the Hck kinase activation assay results indicate that recombinant Vif expressed and purified under denaturing conditions adopts a conformation upon refolding that is compatible with SH3 binding and Src family kinase activation. This biochemically active conformation was next probed...
with HX MS to understand how the protein was folded in solution.

**Vif displayed protection from exchange modulated by zinc**

We next utilized HX MS to probe the solution conformation and dynamics of full-length Vif alone or in the presence of Zn\(^{2+}\). The coordination of Zn\(^{2+}\) by the novel HCCH zinc finger is thought to result in a conformational change in Vif that exposes key residues that interact with Cullin 5.\(^{63}\) To our knowledge, the dissociation constant for the full-length Vif/Zn\(^{2+}\) interaction has not been reported. To ensure that Vif was maximally bound to zinc, we used an elevated concentration of Zn\(^{2+}\) (133 \(\mu\)M) during HX MS labeling. The results of the global conformational HX MS analysis of Vif are shown in Fig. 4. Deuterium incorporation was faster in Vif alone (Fig. 4a) compared to the Zn\(^{2+}\)-bound state (Fig. 4b). The isotope distribution for the +26 charge state of Vif alone shifted to the right of the dotted line (fixed at \(m/z = 870\)) after only 10 s in 2\(H_2\)O, whereas it took more than 5 min for Vif incubated with Zn\(^{2+}\) to cross the line. The change in deuterium is also apparent in deuterium uptake curves (Fig. 4c).

The global HX MS analyses also gave a relative indication as to how much of Vif was highly exposed/not structured versus how much of Vif was highly protected/structured. Vif contains 181 exchangeable backbone amide hydrogen positions. Only approximately 110 of these positions became deuterated in 2 h. Note that these deuteration numbers are reported as relative deuterium incorporation, not as absolute deuterium levels, because we have not corrected for backexchange of deuterium during analysis. With approximately 20–25% deuterium loss due to backexchange, this value of \(\sim 110\) may be as high as 138. Even without backexchange correction, it is clear that a significant number of backbone amide hydrogens (between 43 and 70) did not exchange in the timescale of these measurements. Such results indicate that one or more regions of Vif are significantly protected from hydrogen exchange. Factors that could contribute to the observed amide protection are secondary structural elements, oligomeric complexes, and/or burying of amide positions in the core of the protein. In addition to the information about protection from exchange after 2 h of labeling, incorporation of deuterium at short labeling times also provides information. Backbone amide hydrogens that become deuterated quickly (within the 10-s exchange time point) are primarily those on the surfaces of proteins and/or in highly solvent-exposed regions.\(^{64,65}\) For Vif alone, there were approximately 85–105 amide positions (a higher number takes backexchange into account) that were highly un-
accessibility but retain high protein dynamics and are able to breathe/flex in solution over time to obtain the same amount of deuterium at longer time points (reviewed recently by Morgan and Engen\textsuperscript{66}). These results are in agreement with previous reports in which a synthetic portion of the zinc binding region of Vif underwent a conformational change as a result of zinc coordination.\textsuperscript{67} Our results show that structural changes occurred when full-length recombinant Vif coordinated zinc; these changes might be important for Cullin 5 association\textsuperscript{63} since that is a zinc-finger-dependent interaction.

**The Vif N-terminal region was protected while the C-terminal region was not**

Although the intact HX MS experiments indicated that Vif was partially protected from exchange, they did not indicate where protection occurred in the protein. To localize deuterium incorporation, we utilized pepsin digestion after deuterium labeling,\textsuperscript{68} and the mass of each peptide followed. Note that digestion occurs when the labeling is quenched after the conformational information about the protein at physiological pH has already been captured in the pattern of deuteration. The results of the peptide-level exchange experiments are summarized in Fig. 5 (all deuterium incorporation graphs for the peptides are found in Supplementary Material, Fig. S3). The N-terminal region of Vif displayed the least deuterium incorporation, including residues 1–16 and 40–86, which only became \(\leq 40\%\) deuterated after 20 min in \(\text{H}_2\text{O}\) (Fig. 5a). Interestingly, these highly protected regions consist of the APOBEC3G/F and RNA/DNA binding sites. In contrast, deuterium incorporation into the C-terminal half of Vif was markedly different in that most C-terminal peptides became \(\geq 60\%\) deuterated after only 1 min in \(\text{H}_2\text{O}\). Recall that the C-terminal half of Vif consists of the Vif HCCH zinc finger, viral SOCS box, C-terminal APOBEC3G binding site, Hck binding site, and multimerization domain (Fig. 1).

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**Fig. 5.** Deuterium incorporation at the peptic peptide level. (a) The deuteration map for recombinant Vif. The bars are colored according to the relative percent deuterium incorporation (color code at the top) at each time in deuterium (at the left-hand side). Also indicated are the different regions of Vif and their corresponding biological functions (see also Fig. 1). (b–e) Mass spectra for several Vif peptic peptides. The amino acid sequence of each peptide is shown at the top. Deuteration times (not the same for all peptides: time points along the labeling time course that best illustrate the isotopic distributions and the change with time were chosen) are shown in each panel; UN represents undeuterated protein. The location of the peptides in (b)–(e) is indicated with arrows below (a) and with the m/z of each ion.
The rapid deuteration of this C-terminal region is consistent with the idea that the C-terminal portion of Vif is unstructured in the unbound state. For most of Vif, the isotopic distributions for peptic peptides were characteristic of a traditional single population of one protein conformation in solution (recently well described by Weis et al.). Two examples of this are shown in Fig. 5b and e. The region in Fig. 5b is protected and slowly becomes deuterated, whereas the region in Fig. 5e is not protected and rapidly becomes deuterated. However, several regions of Vif, including a portion of the N-terminal APOBEC3G/F binding site and the HCCH zinc finger, displayed multiple isotopic distributions indicative of multiple conformational states in solution (Fig. 5c and d). Rather than a sharp single isotopic distribution typical of deuterated proteins, there is a much wider distribution. These wide spectra indicate that, in some parts of Vif, there is conformational heterogeneity such that at least two populations of molecules exist that are uniquely protected from exchange. These populations are in equilibrium with each other, interconvert, and—as there is a vast excess of deuterium relative to protium in this type of continuous labeling experiment—eventually become heavily deuterated. Capturing Vif populations existing simultaneously in solution and their interconversion is analogous to the dynamics and labeling for the Hck SH3 domain, as described in Vif Displayed Protection from Exchange Modulated by Zinc. One region of particular interest is the PPLP motif, which has been suggested to be important in Vif multimerization. The deuteration of a peptide that encompassed this region (residues 151–169) is shown in Fig. 5e. After only 10 s in 2H2O, the peptide was highly deuterated, and only a small amount more of deuterium after 2 h of labeling (see also Supplementary Material, Fig. S3). If this region were directly involved in oligomerization as a result of the association of multiple Vif molecules, the amide exchange in this region and in the C-terminus would likely show some protection against deuterium incorporation. No such protection was seen, and it is therefore likely that the role of the PPLP region in Vif oligomerization is not apparent in these in vitro solution measurements. The polyproline type II helix of the PPLP domain could possibly act as a conformational hinge that brings together the regions of Vif required for multimer formation, such as the N-terminus and the C-terminus. This bridging idea is consistent with previous studies in which chemical cross-linking was observed between the N-terminus and the C-terminus of Vif, and with studies showing that when the prolines of the PPLP domain were substituted with alanines (PPLP to AALA), there was decreased (but not abolished) oligomerization.

Zinc coordination stabilizes the Vif HCCH motif

From whole-protein HX MS analysis of Vif (Fig. 4), we determined that zinc binding caused a significant conformational change. To localize the regions of Vif that were differentially deuterated in the presence of zinc, we repeated hydrogen exchange experiments in the presence and in the absence of zinc and digested the deuterium-labeled protein into peptides. The mass spectra of all the peptides were obtained, and deuterium incorporation was determined. The main region that showed changes in deuterium incorporation in the presence of zinc included residues 115–129. As shown in Fig. 6, a peptide covering this region displayed multiple conformational states in solution, apparent in the 10-s time point (see also Fig. 5d). As has become standard practice for bimodal isotopic distributions, two Gaussian distributions were fitted to the 10-s time point using the program Peak Fit (Systat Software, Inc., San Jose, CA). The lower mass distribution (blue) was more protected from amide exchange, likely corresponding to a more folded or structured state of the zinc finger, while the higher mass distribution (red) was more easily deuterated, likely corresponding to an unfolded state(s). Upon addition of zinc, the Vif HCCH peptide (residues 115–129; an overlapping peptide, of residues 115–133, showed a similar deuterium incorporation) was stabilized, and more time was required to achieve deuteration. The average change in the deuterium uptake of Vif alone or in the presence of zinc for residues 115–129 was approximately four deuterons, which is less than that observed in global hydrogen exchange measurements (Fig. 4c). These discrepancies could likely be attributed to several factors, including the absence of peptic peptides in the remainder of the zinc finger (residues 108–114), along with a loss of the deuterium label during pepsin digestion and HPLC separation (backexchange).

The zinc finger in Vif corresponds to residues H108C114C133H139 and is essential for its function. A synthetic peptide encompassing the Vif HCCH (residues 101–142) motif was unstructured in the absence of zinc, however, upon zinc incubation, the motif underwent changes in secondary structure, along with increased tertiary packing near the zinc site, as determined with CD and fluorescence spectroscopy. Our HX MS results suggest that zinc coordination not only alters the secondary structure and tertiary packing of the HCCH motif but also changes the structural dynamics of the HCCH region (Fig. 6). We hypothesize that these changes in dynamics could play a role in an efficient, productive interaction with Cullin 5 and other components of the E3 ligase machinery, which are required for the proper degradation of APOBEC3 enzymes.
Conclusions

The difficulty in producing full-length soluble Vif in suitable quantities for structural and biophysical analyses has hampered advancement in the understanding of Vif biology. Our HX MS analysis of recombinant full-length Vif represents the most detailed conformational study to date. The results are summarized in Fig. 7, where the relative percent unprotected from amide exchange after 1 min in $^{2}$H$_{2}$O is shown for each peptic peptide of Vif. A low percent unprotected (less easily deuterated) is indicative of features such as secondary structural elements, quaternary structure, oligomeric complexes, and/or burying of amide hydrogens such that exchange is slow. A high percent unprotected (more easily deuterated) is indicative of features that are dynamic, are solvent exposed, and most likely do not contain stable higher-order structural elements.

The N-terminal portion of Vif displayed the most protection from amide exchange. If there is any structure in Vif, this region likely contains the majority of it. This region of Vif is essential for interactions with APOBEC3 F/G and DNA/RNA. Interestingly, a portion of the N-terminal part of Vif that binds APOBEC3F/G displays conformational exchange in solution (indicated by striped bars and asterisks; Fig. 7a) (i.e., two simultaneously existing conformations—one that is more protected from exchange than the other). The conformational heterogeneity in this region might be a result of Vif adopting different conformational states for binding to different APOBEC3 proteins, nucleic acids, or other cellular proteins. Alternatively, the more protected conformation of Vif in this heterogeneous mixture of conformations may be the result of oligomerization in these particular regions.

Amide exchange in the C-terminal portion was markedly different from that in the N-terminus in that a majority of the C-terminus became highly deuterated even after short $^{2}$H$_{2}$O incubation times. This behavior suggests that the majority of the C-terminus is solvent exposed and likely unstructured in the absence of the E3 ligase machinery and zinc. Our results for full-length Vif are consistent with several reports in which synthetic peptides based on the C-terminus of Vif were found to be unstructured in solution.67,69 The lack of structure in the absence of interacting partners in the C-terminus of Vif likely allows this region to adopt different conformations in solution, enabling Vif to associate with multiple binding partners such as Cullin 5, Elongin BC, Hck, and APOBEC3 proteins.

It was important in these structural studies of Vif to use recombinant protein that was as biochemically functional as possible. As Vif has no enzymatic activity of its own, and instead functions by binding to other proteins and protein complexes, binding to partner proteins was the assay that had to be used to test for biochemical functionality. We previously showed that recombinant Vif prepared exactly as described in the current work could effectively interact with the Elongin BC complex.16 We now show that the same recombinant refolded Vif was capable of binding to the tyrosine kinase Hck via its SH3 domain and that the interaction was specific for the known Hck SH3 PxxP binding motif (Fig. 3). We showed that recombinant Vif potently activates Hck
and other Src family members both in vitro and in a defined cell-based system (yeast). There have only been a few other reports of Hck/Vif interaction in the literature. The ability of Vif to alter kinase activity supports a meaningful role for Vif-induced regulation of Src family kinases, perhaps through a connection between kinase activation and cell type restriction of APOBEC3 proteins. Activation of Src family tyrosine kinases seems to be an inherent biochemical property of recombinant Vif, raising the possibility that compounds targeting the Vif/SFK interaction may exhibit antiretroviral activity. A similar approach has been successfully applied to Nef/Hck interaction, leading to the identification of small molecules that inhibit Nef-dependent HIV replication.

Our HX MS results for full-length Vif, along with recent reports in the literature on synthetic Vif peptides, provide molecular insights into the structure of this essential HIV-1 viral accessory protein. Figure 7b depicts a cartoon model of Vif that is consistent with all of our data. Vif likely contains a folded core domain in the N-terminal portion of the protein that acts as the adaptor region for APOBEC3F/G, and for interaction with Src family kinases. These binding regions may or may not require structure to bind to their targets effectively.

Materials and Methods

Protein expression and purification

Full-length HIV-1 Vif (from the HIV strain HXB2) was overexpressed from a codon-optimized pET28b vector (a gift from Dana Gabuzda at the Dana Farber Cancer Institute). E. coli strain Rosetta 2 DE3 pLysS was transformed with the pET28b plasmid, and the cells were grown to OD600 values of 0.6–1.0. Protein expression was induced by addition of 100 μM IPTG and allowed to proceed for 4 h at 37 °C. Cells were harvested by centrifugation and lysed in 10 mL of lysis buffer (6 M guanidine hydrochloride and 0.1 M sodium phosphate, pH 8.0). The lysate was centrifuged at 20,000g for 40 min at

Fig. 7. Summary of results and model of Vif. (a) Summary of deuterium incorporation into Vif after 1 min in 2H2O. Each bar represents a Vif peptic peptide. Shown is the relative percent unprotected for each region of Vif. The residues that each peptide encompasses are indicated in the center of each bar, and the maximum number of exchangeable amide hydrogens is indicated by the number in the top right of each bar. Relative percent unprotected was determined by dividing the relative deuterium incorporation at 1 min by the maximum possible exchangeable hydrogens. The peptides that displayed multiple populations in HX MS are indicated by stripes and asterisks (**). The relative percent protection for these peptides was calculated using the average deuterium incorporation across the entire isotopic distribution of the multiple populations observed. The N-terminal portion showed the lowest amount of deuterium incorporation (most protection from amide exchange) and therefore likely contains the majority of structural elements, while the C-terminal portion showed the highest amount of deuterium incorporation (least protection from amide exchange), consistent with a dynamic and unstructured protein. (b) Model of HIV-1 Vif based on the HX MS results. The N-terminal portion of Vif has structure, while the C-terminal portion has none. The C-terminal portion of Vif becomes partially organized upon binding zinc and components of the E3 ligase machinery. Parts of this figure were adapted from Mehle et al.
4 °C to remove the insoluble component; the supernatant was incubated with Ni²⁺-NTA agarose (Qiagen, Valencia, CA) and mixed end over end for 30 min at room temperature. Ni²⁺-NTA agarose was washed three times with 10 mL each of lysis buffer. Vif was eluted from the Ni²⁺-NTA resin using a step gradient of lysis buffer with decreasing pH values (pH 7.0, pH 6.5, pH 6.0, pH 5.5, and pH 5.0). All fractions of pH values ≤ 5.5 were checked for protein using mass spectrometry and SDS-PAGE. Fractions containing Vif were pooled and diluted, so the concentration was below 35 μM (as determined by Bradford assay) from Bio-Rad, Hercules, CA. The diluted protein was dialyzed against Vif buffer [20 mM Hepes, 150 mM NaCl, 1 mM DTT, and 10% glycerol (pH 7.0)] for 1.5 h each, with Vif buffer containing 3.0 M, 1.5 M, 0.75 M, 0.21 M, and 0 M guanidine HCl, respectively. A second dialysis with 0 M guanidine HCl was conducted overnight to ensure the complete removal of denaturant. The final dialyzed material was rechecked with mass spectrometry and stored at −80 °C.

Human wild-type Hck SH3 was overexpressed and purified as described previously. The coding sequence for the human Hck SH3 domain with the inactivating Trp93-to-alanine (W93A) mutation was PCR amplified from a full-length Hck template bearing this mutation. The Trp93-to-alanine (W93A) mutation was PCR amplified for the human Hck SH3 domain with the inactivating Trp93-to-alanine (W93A) mutation was PCR amplified from a full-length Hck template bearing this mutation. The resulting cDNA fragment was subcloned into the bacterial expression vector pET28b using the XhoI and HindIII restriction sites. Hck SH3 W93A was overexpressed in E. coli Rosetta 2 DE3 pLysS cells as described above, and the soluble protein was purified with Ni-NTA affinity chromatography. The affinity tags were removed from the SH3 domains (wild type and W93A) by overnight cleavage with thrombin, followed by dialysis with Vif buffer and storage at −80 °C. Protein concentrations were determined by Bradford assay, and purity was verified with SDS-PAGE and electrospray mass spectrometry.

**CD measurements**

CD measurements were taken with a J-715 CD spectrometer (Jasco Instruments, Easton, MD) with a path length of 1 mm at 20 °C. For analysis of refolded Vif, the buffer was composed of 20 mM Hepes, 150 mM NaCl, 1 mM DTT, and 10% glycerol (pH 7.0). Denatured Vif was analyzed in the same buffer containing 8 M guanidine HCl (pH 7.0). Deconvolution of the CD spectra and assignment of Vif (30 μM) was thawed from −80 °C and incubated at 4 °C for 5 min before labeling. The Vif solution was diluted 15-fold with 20 mM Hepes, 150 mM NaCl, 1 mM DTT, and 99.99% ²H₂O (pD = 7.0) at 20 °C. For experiments that included zinc, Vif was equilibrated in the presence of 2 mM ZnCl₂ before labeling; upon 15-fold dilution with labeling buffer, the zinc concentration was 133 μM. The labeling reaction was quenched at various times by adjusting the pH to 2.6 at a 1:1 ratio of a solution containing 0.8 M guanidine HCl, 0.8% formic acid, and 100% H2O. For SH3 binding experiments with recombinant Vif, wild-type Hck SH3 or W93A Hck SH3 was diluted to 6 μM and incubated with a 5-fold molar excess of Vif (30 μM) for 30 min at 4 °C. The mixture was labeled and quenched exactly the same as described for Vif alone.

**Deuterium labeling**

Vif (30 μM) was thawed from −80 °C and incubated at 4 °C for 5 min before labeling. The Vif solution was diluted 15-fold with 20 mM Hepes, 150 mM NaCl, 1 mM DTT, and 99.99% ²H₂O (pD = 7.0) at 20 °C. For experiments that included zinc, Vif was equilibrated in the presence of 2 mM ZnCl₂ before labeling; upon 15-fold dilution with labeling buffer, the zinc concentration was 133 μM. The labeling reaction was quenched at various times by adjusting the pH to 2.6 at a 1:1 ratio of a solution containing 0.8 M guanidine HCl, 0.8% formic acid, and 100% H2O. For SH3 binding experiments with recombinant Vif, wild-type Hck SH3 or W93A Hck SH3 was diluted to 6 μM and incubated with a 5-fold molar excess of Vif (30 μM) for 30 min at 4 °C. The mixture was labeled and quenched exactly the same as described for Vif alone.

**Intact protein mass analysis**

Immediately after the exchange quench, protein samples were injected into a Shimadzu SCL-10A VP HPLC flowing water containing 0.05% formic acid (pH 2.6) at 50 μL/min, coupled to a Waters LCT premier mass spectrometer with a standard electrospray interface.
Proteins were eluted directly into the mass spectrometer with a gradient of 15–98% acetonitrile (containing 0.05% formic acid, pH 2.6) in 3.5 min. The injector, the column (an Alltech analytical in-line guard column packed with POROS 20-R2 reversed-phase media; PerSeptive Biosystems), and all associated tubing were kept at 0 °C to minimize backexchange. All experiments were conducted under identical conditions so that the relative deuterium levels could be compared. Deuterium levels were not corrected for backexchange and are therefore reported as relative deuterium levels. Intact mass spectra were deconvoluted using the software MagTran. The intact protein relative deuterium uptake was calculated by subtracting the centroid mass of an undeuterated control from the centroid mass of deuterium-labeled samples. All intact HX MS experiments were conducted at least twice, and the results were averaged.

**Peptide mass analysis**

Using a Shimadzu SCI-10A VP HPLC, we injected protein samples into an immobilized pepsin digestion column (2.1 mm×50 mm stainless-steel column packed with immobilized pepsin; POROS-20AL beads; PerSeptive Biosystems) flowing 0.05% formic acid (pH 2.6) at 20 °C at 200 μL/min. Peptic peptides produced in the pepsin column were trapped on a Michrom Bioresources (Auburn, CA) peptide micropeptide trap at 0 °C and desalted for 3 min before separation using a POROS 20 R2 (PerSeptive Biosystems) column. The column was 0.20 mm×100 mm and operated at 0 °C at a flow rate of 50 μL/min. A 9-min 8–40% acetonitrile gradient (both mobile phases contained 0.05% formic acid, pH 2.6) was used to elute the peptides directly into a Waters QTOF API US mass spectrometer with standard electrospray interface. A mass accuracy of <5 ppm was maintained through continuous lock mass correction carried out using Glu fibrinogen peptide. The Excel-based software program HX-Express was used to process the data. The deuterium uptake for each peptide was determined by subtracting the centroid mass of each undeuterated control from the centroid mass of deuterium-labeled samples. All peptide-level HX MS experiments were conducted at least twice, and the results were averaged. In this experimental setup, the error of measuring the deuterium incorporation was less than ±0.50 Da. All peptic peptides were identified by a combination of exact mass analysis tandem mass spectrometry and Waters Identity software. All peptide-level HX MS experiments were conducted at least twice.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.04.053

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**References**


Supplemental material for:

On the solution conformation and dynamics of the HIV-1 Viral Infectivity Factor

Sean R. Marcisin, Purushottam S. Narute, Lori A. Emert-Sedlak, Marek Kloczewiak, Thomas E. Smithgall, and John R. Engen

Figure S1.

Activation of the Src-family tyrosine kinases Hck, Lyn, and Lck by HIV-1 Vif. Near full-length Hck, Lyn and Lck were expressed in Sf9 insect cells in their downregulated, YEEI-tailed forms and purified to homogeneity as described elsewhere \(^1\); \(^2\). Recombinant purified Vif was combined with each Src-family member at the molar ratios shown, and kinase assays were performed using the FRET-based Z’-Lyte assay and Tyr-2 peptide substrate according to the manufacturer’s instructions (Invitrogen). Assays (10 \(\mu\)L final) were performed in quadruplicate in 384-well plates with ATP and Tyr-2 peptide substrate concentrations of 50 \(\mu\)M and 2 \(\mu\)M, respectively. Kinases were pre-incubated with Vif for 30 min, followed by incubation with ATP and the Tyr-2 peptide substrate for 1 h. Following reaction quench, substrate fluorescence was assessed on a Gemini XS microplate spectrofluorometer (Molecular Devices). Each reaction condition was monitored in quadruplicate, and results are presented as mean percent of control peptide phosphorylation ± S.D. All three of the kinases tested required sub-stoichiometric Vif:kinase ratios for kinase activation. The data shown for Vif-induced Hck activation is the same as that presented in Figure 3C, and is included here for reference.
**Figure S2.**

Vif activates the Src-family kinases Hck, Lyn and Lck in yeast cells. Yeast cultures were transformed with galactose-inducible expression plasmids for HIV Vif, Hck-YEEI, Lck-YEEI, and Lyn-YEEI either alone or in the combinations shown. (YEEI refers to the tail modification present in each of these kinases that causes downregulation in the absence of Csk; 1,3). Yeast cultures transformed with active, wild-type (wt) forms of each Src-family member were also included as positive controls. Liquid cultures were normalized for cell density, and equal volumes were spotted on galactose-agar plates at increasing dilutions and incubated at 30 °C for three days. Scanned images of the plates are shown at the top, in which the yeast patches appear as dark circles. Note that co-expression of Vif with each Src-family member inhibits yeast cell growth, as reflected by loss of the dark spot and indicative of kinase activation. Expression of Vif and Src-family kinase proteins were verified by immunoblotting along with actin as a loading control. Yeast protein extracts were also immunoblotted with antiphosphotyrosine antibodies (bottom) as a measure of cellular kinase activity; the strong signal observed with wild-type Lyn expression is due to the high level of Lyn kinase expression in this culture.

**Figure S3.**

Deuterium incorporation graphs of all the peptic peptides followed in Vif HX MS. Relative deuterium uptake (Da) is plotted vs. time in $^2$H$_2$O. The Y-axis of each graph is set to the maximum possible deuterium incorporation of each indicated peptide. The primary structure of each peptide is indicated as well as the amino acid residue numbers.
The peptide data for Vif alone is shown in blue while the peptide data for Vif with zinc is shown in pink. The data shown here are the average of two replicate labeling experiments. The error of peptide HX MS measurements with this experimental setup was ± 0.50 Da as determined with replicate analysis of peptides standards and prior HX MS work with this instrumentation.4

REFERENCES


Figure S1
Figure S2
Figure S3
APPENDIX II

Publication

Hydrogen exchange mass spectrometry: what is it and what can it tell us?

Sean R. Marcisin · John R. Engen

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Abstract Proteins are undoubtedly some of the most essential molecules of life. While much is known about many proteins, some aspects still remain mysterious. One particularly important aspect of understanding proteins is determining how structure helps dictate function. Continued development and implementation of biophysical techniques that provide information about protein conformation and dynamics is essential. In this review, we discuss hydrogen exchange mass spectrometry and how this method can be used to learn about protein conformation and dynamics. The basic concepts of the method are described, the workflow illustrated, and a few examples of its application are provided.

Keywords Deuterium · Protein mass spectrometry · Protein dynamics · Protein conformation

Introduction

Proteins play a pivotal role in most biological processes, including DNA replication, cell division, cell death, immune response, and cellular signal transduction. Many of these processes are thought to be carried out by protein machines containing at least 10 or more proteins [1]. To fully understand how proteins drive and contribute to basic biological and biochemical events, techniques that probe the fundamental properties of proteins are necessary. Not only must these methods provide information about protein function, they must also help reveal how function is tied to protein conformation and dynamics. In the past 60 years or so, there have been profound advances in the techniques for protein analysis, including such Nobel Prize-winning methods as nuclear magnetic resonance (NMR), X-ray crystallography, small angle X-ray scattering, and cryo-electron microscopy. As with everything, each of these techniques has advantages and disadvantages for studying protein conformation and dynamics, and often a combination of methods is required for full protein understanding.

An orthogonal biophysical technique that has also seen profound advances in the last 20 or so years is Hydrogen Exchange monitored by Mass Spectrometry (HX MS). HX MS probes solution conformation so crystallization is not required, it requires very little sample (500–1,000 picomoles for an entire experiment), it is amenable to studying proteins that are hard to purify or that can only be handled at low concentrations (as low as 0.1 μM), and it can reveal changes to conformation and dynamics on a wide timescale (for a recent review and perspective, see Ref. [2]).

Topics

Overview of hydrogen exchange: what is it?

Hydrogen exchange mass spectrometry exploits a fundamental chemical reaction unique to hydrogens found in proteins (for a historical perspective, see Ref. [3]). Certain hydrogens in proteins are in continuous exchange with the hydrogens in solution. If an aqueous, all-H2O solvent is
replaced with an isotope of hydrogen that has distinctive spectroscopic properties, then one can follow this exchange process. For most modern HX experiments, deuterated or “heavy” water (D₂O) is used. In particular, the hydrogen bonded to the backbone nitrogen (also referred to as the backbone amide hydrogen) is useful for probing protein conformation. Figure 1a shows the location of the backbone amide hydrogens relative to other chemical groups within the protein. Note that every amino acid except for proline has a backbone amide hydrogen, meaning that there is a sensor at every amino acid along the length of the protein chain. While other hydrogens on side chains can also undergo exchange, the rate of side chain exchange is generally much faster than that of the backbone positions and any deuterium that is incorporated in non-backbone positions reverts back to hydrogen later in the experiment (described below).

There are various factors that affect hydrogen exchange in proteins [4]. The four primary factors are pH, temperature, solvent accessibility, and hydrogen bonding. Temperature and pH can be controlled experimentally meaning that the rate and location of exchange then becomes a function of hydrogen bonding and solvent accessibility. While amide hydrogens of fully solvent-exposed peptides at pH 7 exchange very rapidly with rates of 10-1,000 s⁻¹ (depending on solvent conditions, etc.) [5], in folded proteins amide hydrogens display a variety of exchange rates depending on their position within the protein and whether they are involved in intramolecular hydrogen bonding. Figure 1b illustrates how solvent accessibility and hydrogen bonding relate to exchange in proteins. Regions that are highly dynamic and solvent-exposed (like the loops connecting the alpha helices) will exchange rapidly while regions that are less dynamic (i.e., “rigid”) and/or involved in hydrogen bonding networks or buried within the interior of the protein (such as β-sheets or α-helices) will exchange slower. In folded proteins some amide hydrogens exchange quickly while others exchange much slower on timescales from minutes to months [4, 6]. Note also that the backbone amide hydrogens are the ones participating in the hydrogen bonds that hold α-helices and β-sheets together. If there is a change to the solvent accessibility or the hydrogen bonding network of a protein, the rate and location of deuterium incorporation can be altered.

Monitoring hydrogen exchange

The different chemical properties of hydrogen and deuterium allow several biophysical techniques to distinguish between the two isotopes. If deuterium incorporation into proteins is monitored with NMR, for example, the amide proton peaks (from hydrogen) disappear as the protein becomes deuterated because deuterium is NMR silent. As the mass of hydrogen is 1.0078 Da and the mass of deuterium is 2.0141 Da, deuterated proteins will have a larger mass than non-deuterated proteins [7]. All that remains to convert deuterium information into conformational information is to determine how fast a protein is deuterated and where the deuterium goes.

A general workflow for a hydrogen exchange experiment monitored with mass spectrometry is shown in Fig. 2. This figure shows the continuous labeling experiment, which is the most common [8]. Protein solutions are initially equilibrated at room temperature, physiological pH, and in all-H₂O buffer. A dilution of 10- to 20-fold into the identical buffer except with all D₂O initiates the labeling process. The excess deuterium insures the exchange kinetics favor labeling from hydrogen to deuterium [9]. The labeling is allowed to proceed for various amounts of time (e.g., 10 s, 1 min, 20 min, 8 h) and then the labeling is quenched by adjusting the pH of the sample to 2.5 and lowering the temperature to 0 °C. These quench conditions
ensure retention of the deuterium label for MS analysis by decreasing the rate of amide exchange approximately five orders of magnitude \([6, 10]\). The quenched protein sample can then be sprayed directly into a mass spectrometer (using liquid chromatography as the interface) to determine the mass of the intact protein. Measuring the mass of the whole protein gives global information about the protein(s) being studied but does not locate where the deuterium is within the protein. Proteins can also be digested post-quench but prior to chromatography. This digestion must be conducted under quench conditions to ensure retention of the deuterium label; therefore, acid proteases such as pepsin must be used \([10, 11]\). Note that all peptic peptides must also be identified, typically done in separate MS/MS experiments. With a digestion experiment, deuterium can be localized within the short peptides produced by the digestion. The location cannot be refined to single amino acids without the use of more sophisticated mass spectrometry methods \([12]\).

What can hydrogen exchange tell us?

HX MS can provide very useful information, including: how conformation changes upon binding, protein folding and unfolding pathways, glimpses into the structure of proteins that will not crystallize or are not amenable to NMR, which regions of a protein are solvent-exposed and dynamic, location and properties of binding sites and surfaces, dissociation constants and measures of protein stability under various conditions. The raw mass spectra of the mass of the protein, or each peptide from the digestion, are converted into a deuterium uptake plot where the level of deuterium is plotted versus the labeling time. Deuterium uptake plots for both intact analysis and peptide analysis contain information about how solvent-exposed and dynamic a protein is in solution. For a detailed explanation of all the information that can be derived from deuterium uptake plots see Ref. \([13]\).

To illustrate how HX MS data can be revealing, consider Fig. 3 which shows examples of deuterium uptake plots for two proteins under different hypothetical situations. In Fig. 3a, the use of HX MS to probe structural effects as a result of small molecule/inhibitor binding is represented; Fig. 3b illustrates results for protein–protein interactions in a hexameric protein assembly. In the intact analysis in Fig. 3a(i), when the protein is incubated with an inhibitor, less deuterium is incorporated over the course of the labeling experiment. The differences in deuteration appear at later times in the exchange process, which indicates changes in solution dynamics as a result of inhibitor binding \([14]\). The overall motions of the protein were decreased, that is its structure was rigidified and it could not flex as well in solution, hence less deuterium was incorporated. In order to identify which specific parts of the protein were changed upon binding, the pepsin digestion experiment can be performed prior to MS

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**Fig. 2** Workflow of a typical HX MS experiment. Protein samples are equilibrated at the desired temperature and pH, in a buffer compatible with the protein. Protein solutions are then diluted (typically 10-fold or more) with the identical buffer containing 99.9% D_2O instead of H_2O. The exchange reaction proceeds for various amounts of time and is quenched by lowering the pH to 2.5 and the temperature to 0 °C. The pH and temperature adjustment reduces the amide exchange rate to its minimum. Deuterated, quenched protein can then be either directly injected into a mass spectrometer for mass analysis or digested with an acid protease prior to liquid chromatography and mass analysis. The mass spectra are analyzed and the uptake of deuterium over time determined and plotted, either for the intact protein, or for each of the peptic peptides.
HX MS reports on the effects of protein binding: a protein plus small molecule inhibitor or b protein–protein interactions as a result of quaternary structure formation. In a, analysis of the intact, undigested protein (i) can reveal if there are any global conformation and/or dynamic changes as a result of inhibitor binding. In this example, the inhibitor causes a reduction in deuterium incorporation (red curve). By digesting the deuterated protein with a protease, one can observe the location of the differences (ii and iii). Residues 20–35 of this protein (ii) undergo a reduction in deuterium incorporation upon inhibitor binding while residues 70–89 (iii) do not. The location of each peptide is known (structural insets). In b, deuteration of the monomeric subunit of a hexameric protein was compared with deuteration of the same monomer when part of the hexameric assembly found in vivo. Intact protein analysis (i) indicates protection from exchange in the hexamer and the location was determined to be primarily residues 10–28 (ii) and not residues 50–68 (iii).

In addition to probing structural changes in proteins as a result of ligand/inhibitor binding, HX MS can be used to study many other types of protein interactions, including protein–protein interactions. Since the majority of cellular processes are orchestrated by multiple proteins and large macromolecular protein complexes, being able to study the structural consequences of these interactions is extremely important. Figure 3b illustrates an example of how HX MS could be used to study protein–protein interactions. In this hexameric protein, exchange can be compared in the isolated monomer versus the monomer in the assembled hexamer (Fig. 3b(i)). Then, with the use of pepsin digestion, the specific regions affected by complex formation can be determined (residues 10–28 are involved in hexamer formation, residues 50–68 are not; Fig. 3b(ii and iii)).

One could imagine many protein–protein interactions in which determining the effects and location(s) of changes in HX upon binding would be useful. For example, HX MS has been used to study the structural changes induced by pH changes in the capsid protein of the brome mosaic virus [15] and it has been used to investigate conformational changes in the HIV-1 capsid protein as a result of HIV assembly and maturation [16]. The large size and complexity of viral capsids make them challenging to study by many structural means, particularly when detailed information about conformational changes is desired. HX MS, however, can allow access to such information and has therefore become important as a tool for probing large proteins and protein complexes.

HX MS is also sensitive to populations of molecules in solution. If multiple populations exist that are different structurally, different amounts of deuterium will be incorporated into each population and this can be observed in the raw mass spectra [17], as illustrated in Fig. 4. If the refolding rate ($k_{-1}$) of unfolded and exposed regions of a
protein is slower than the deuterium labeling rate (Fig. 4a), the unfolded species will have sufficient time to get highly deuterated and therefore have a higher mass than the folded species [4, 18, 19]. D₂O is present in vast excess such that once an individual protein molecule has transitioned to the unfolded form and become deuterated, it is a permanent member of the unfolded species peak (red, bottom of Fig. 4a). The conversion of the folded to the unfolded species occurs at a rate indicative of the rate of protein unfolding in solution. This type of exchange behavior is rare for proteins, but when observed, can serve as an extremely powerful tool for studying protein–protein interactions. A good example comes from the SH3 domains of the Src family of tyrosine kinases [20]. Data from the HX of the SH3 domain of the Lyn kinase alone (Fig. 4b) and in complex with the HIV accessory protein Nef (Fig. 4c) indicate multiple populations existing in solution. The Nef protein has been shown to interact with different members of the Src family of tyrosine kinases, including the Lyn kinase, and to disrupt cellular signaling to enhance viral infection [21]. In HX MS of Lyn SH3 alone, the unfolding rate occurs with a half-life of approximately 12 min but when the Lyn SH3 domain is bound to Nef, the unfolding dynamics of Lyn SH3 are slowed to a half-life of longer than 30 min. From this type of data, one can extract relative binding affinities [22], or determine when and if certain sequences are able to associate intramolecularly [23, 24].

**Outlook**

Because the function of proteins is dictated by their structure and movements in solution, any biophysical technique which allows for the molecular investigation of proteins will be invaluable to aiding in our understanding of these molecular machines. Amide hydrogen exchange monitored by mass spectrometry is just one such technique for studying proteins. To become robust and high throughput, HX MS faces some challenges. Development of robotics and total automation of sample handling and data processing will make the method more amenable to high-throughput types of studies. Advances in liquid chromatography and mass spectrometry will also continue to improve the HX MS experiment. A major limitation in terms of chromatography during HX MS experiments is that separation must be done at 0 °C where chromatographic efficiency in traditional HPLC is relatively poor. New separation media and the use of UPLC [25] are

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**Fig. 4** Using hydrogen exchange to monitor the HIV-1 Nef:Lyn SH3 complex. a An advantage of HX MS over some other biophysical techniques is the ability to detect different protein populations in solution. In this example, the two populations appear in the mass spectra: one representing the folded state (blue distribution) and the other representing the unfolded state (red distribution). The appearance of two distributions occurs when the rate of interconversion of the two populations (i.e., folded and unfolded) is slower than the amide exchange rate (recently discussed in Ref. [18]). If a molecule unfolds, it will become totally deuterated, hence the higher mass. b,c The Src Homology 2 (SH3) domain of the Lyn kinase shows a bimodal pattern in intact protein HX MS (+6 charge state shown). This pattern indicates partial, cooperative unfolding as described in a. One of the labeling timepoints captures the populations at an approximate 60:40 ratio (folded to unfolded), indicated by the asterisk. The actual 50:50 population point (or 1/2 for the unfolding reaction) is observed 12 min after deuterium labeling begins. In c, the unfolding is shown for the +6 charge state of intact Lyn SH3 bound to the HIV-1 accessory protein Nef [22]. Notice that while the pattern of the bimodal distribution of folded and unfolded species appears similar to that shown for Lyn SH3 (b), the time at which it appears during the deuterium labeling time course is much longer in the bound form (c). The 60:40 ratio is again marked with an asterisk and occurs at a much later time in the labeling timecourse (30 mins). Data such as these give insight into the molecular mechanisms which govern enhancement of HIV replication through the Nef:Lyn complex.

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addressing this issue and also allowing for analysis of bigger and bigger proteins and protein systems [26]. The large amount of data produced during hydrogen exchange experiments have historically made data processing time-consuming. Reducing the data to a form that is easily understood in terms of conformation for a given protein can be arduous. Recent software developments (e.g., HYDRA [27], HD Desktop [28], and TOF2H [29]) significantly reduce the burden of HX MS data analysis although much more work needs to be done in this area. More sensitive mass spectrometers will also improve HX MS. One important parameter of an HX MS experiment is obtaining the protein to be studied. If proteins are rare, difficult to overexpress or obtain in the concentrations needed for some biophysical methods, or just otherwise uncooperative, there may be significant problems obtaining suitable material for analysis. Luckily, HX MS has some of the lowest requirements of any of the biophysical techniques that can provide conformational data, thereby providing access to some of these proteins that are difficult to deal with, particularly the estimated 30% of eukaryotic proteins that contain unstructured regions [30]. We envision that conformational data on many proteins that were considered impossible to analyze will be provided by HX MS in the future.

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References

APPENDIX III

Publication

Molecular Insight into the Conformational Dynamics of the Elongin BC Complex and Its Interaction with HIV-1 Vif

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Keywords: hydrogen exchange mass spectrometry; protein conformation; viral SOCS box; APOBEC3; E3 ubiquitin ligase

The human immunodeficiency virus type 1 virion infectivity factor (Vif) inhibits the innate viral immunity afforded by the APOBEC3 family of cytidine deaminases. Vif targets the APOBEC3 family for poly-ubiquitination and subsequent proteasomal degradation by linking the Elongin-BC-dependent ubiquitin ligase complex with the APOBEC3 proteins. The interaction between Vif and the heterodimeric Elongin BC complex, which is mediated by Vif's viral suppressor of cytokine signaling box, is essential for Vif function. The biophysical consequences of the full-length Vif:Elongin BC interaction have not been extensively reported. In this study, hydrogen exchange mass spectrometry was used to dissect the Vif:Elongin BC interaction. Elongin C was found to be highly dynamic in the Elongin BC complex while Elongin B was much more stable. Recombinant full-length Vif interacted with the Elongin BC complex in vitro with a $K_d$ of 1.9 $\mu$M and resulted in observable changes in deuterium uptake in both Elongin C and B. Upon binding to Elongin BC, no significant global conformational changes were detected in Vif by hydrogen exchange mass spectrometry, but a short fragment of Vif that consisted of the viral suppressor of cytokine signaling box showed decreased deuterium incorporation upon Elongin BC incubation, suggesting that this region folds upon binding.

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Introduction

The Elongin BC complex is an essential component of the Elongin-BC-dependent E3 ubiquitin ligase complex. Human Elongin B and C were originally identified as part of the heterotrimeric transcription factor Elongin ABC complex, which promotes elongation during transcription by suppressing pausing of RNA polymerase II. Elongin B and C also have the ability to form the heterodimeric Elongin BC complex, which associates with cellular proteins that contain a suppressor of cytokine signaling (SOCS) box motif. Upon binding SOCS-containing proteins (such as SOCS1–7, CIS), the Elongin BC complex contributes to modulation of intracellular responses from various cytokines by bridging SOCS family of proteins and their specific cytokine-related targets with machinery of the E3 ubiquitin ligase for proteasomal targeting and subsequent degradation.

Human immunodeficiency virus type 1 (HIV-1) virion infectivity factor (Vif) exploits the Elongin-BC-dependent E3 ubiquitin ligase complex for the targeted degradation of specific cellular antiviral factors such as APOBEC3G. Vif is an accessory HIV gene product that is essential for viral replication.

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Abbreviations used: Vif, virion infectivity factor; HIV-1, human immunodeficiency virus type 1; SOCS, suppressor of cytokine signaling; HX, hydrogen exchange; MS, mass spectrometry; PDB, Protein Data Bank; VHL, Von Hippel-Lindau.
antiviral activity of cytidine deaminases of the APOBEC3 family by directing them to proteasomal degradation through an Elongin-BC-dependent E3 ubiquitin ligase.\textsuperscript{8,10–13} Mutations in Vif that prevent association with the Elongin BC complex disrupt proteasomal targeting and degradation of the APOBEC3 proteins (such as A3G and A3F) resulting in a non-productive HIV infection.\textsuperscript{14–16} The importance of the Elongin-BC-dependent Vif ubiquitin ligase complex for destruction of innate cellular immunity against HIV makes understanding the Vif:Elongin BC complex, both structurally and biophysically, extremely important.

To date, it has been difficult to characterize the Vif:Elongin BC complex due to difficulties in producing soluble full-length Vif and other components of the E3 ligase complex in the relatively high concentrations required for traditional biophysical analysis. In addition, conformational changes in Elongin C when it is part of the Elongin BC complex have not been extensively studied, likely due to the inability of cellular SOCS box sequences (SOCS1–7) to associate with the Elongin BC complex \textit{in vitro}.\textsuperscript{17} The existing biophysical data for Vif and Elongin BC are, nonetheless, enlightening. The crystal structure of Elongin BC with a fragment (residues 140–156) of Vif (HXB3)\textsuperscript{12} provides insight into how the novel SOCS box in Vif associates with Elongin C in the Elongin BC complex but does not reveal conformational dynamics and biophysical consequences of this interaction or report on conformational properties of the remainder of Vif in the complex. Recently, Reingewertz \textit{et al.} prepared a synthetic version of the C-terminal domain of Vif and found experimentally that it was unfolded in the absence of interacting proteins such as the Elongin BC complex\textsuperscript{16} and unfolded based on computation.\textsuperscript{17} Upon incubation with an Elongin C peptide, no structural changes in the C-terminal domain of Vif were detected.\textsuperscript{16} Elongin C, on the other hand, underwent significant conformational changes when it was incubated (in the absence of Elongin B) with either sequences containing a SOCS box motif [such as Von Hippel–Lindau (VHL)] or Elongin A.\textsuperscript{18}

In the present study, hydrogen exchange (HX) monitored by mass spectrometry (MS) was used to investigate the conformational dynamics of the Elongin BC complex and the interaction with Vif \textit{in vitro}. HX MS is a biophysical technique that is amenable to studying proteins and protein complexes where only small quantities of material are available.\textsuperscript{19} HX MS therefore seemed highly suited to the study of Vif and Elongin because it does not require large amounts of protein at high concentration but is still very sensitive to conformational changes as a result of protein–protein interactions.\textsuperscript{20–21} Using HX MS, we determined that Elongin C was dynamic in solution even when associated with Elongin B. On the other hand, relative to Elongin C, Elongin B was conformationally stable in the Elongin BC complex and only the C-terminal tail was highly dynamic. In experiments where Elongin BC bound to recombinant full-length HIV-1 Vif, there were significant changes to deuterium incorporation in Elongin C localized to the Vif binding interface. The stoichiometry of the wt Vif:Elongin BC interaction was found to be 1:1 and of moderate affinity and required no additional cellular components for assembly \textit{in vitro}. The Vif:Elongin BC interaction was specific and required the C-terminal viral BC box of Vif.

Results and Discussion

\textbf{HIV-1 Vif interacts with the Elongin BC complex and alters Elongin C conformation}

Although members of the human cellular SOCS protein family (SOCS1–7, CIS) bind to the Elongin BC complex when co-expressed \textit{in vitro}, cellular SOCS box proteins have been reported to not bind Elongin BC \textit{in vitro}.\textsuperscript{15} HIV-1 Vif is an exception, as the affinity of short fragments of Vif and the Elongin BC complex \textit{in vitro} was recently reported by two different groups,\textsuperscript{22,23} the affinity of full-length HIV-1 Vif for the Elongin BC complex has not been reported. To investigate whether full-length recombinant HIV-1 Vif could bind Elongin BC \textit{in vitro}, we utilized HX MS.

HX at backbone amide hydrogen positions is sensitive to protein complex formation in that exchange rates are generally slower in proteins that are bound to one another.\textsuperscript{24} The hydrogens at backbone amide positions in proteins are in continuous flux with hydrogens in the solvent. By replacing ordinary aqueous solvent containing hydrogen with aqueous solvent containing 99.9 mol\% deuterium ($^2$H, an iso-
HX rates are changed. Changes in protein dynamics may occur at the binding interface or elsewhere as a result of structural changes that can be communicated through the protein molecule (e.g., Refs. 26 and 27). Complex formation may be probed with MS simply by measuring the amount of deuterium incorporated into members of a protein complex when alone and comparing the results to deuterium levels for the same protein members when part of a complex.

The ability of full-length recombinant Vif to associate with the Elongin BC complex was assayed with HX MS by incubating Elongin BC with a fourfold molar excess of full-length Vif and monitoring the deuterium incorporation in both Elongin B and C. Figure 1 shows the +9 charge state of Elongin C in the Elongin BC complex as it became deuterated with or without Vif. In the presence of Vif, the mass increase of Elongin C was suppressed as a result of complexation. The deuterium uptake curves for Elongin C (Fig. 1c) show that a large portion of Elongin C, nearly 20 residues, was protected from HX upon complexation with Vif. Several factors could contribute to such protection including stabilization of the Elongin C structure or the protection of backbone amide hydrogens in Elongin C by the presence of Vif. In addition to monitoring Elongin C, the conformational affects of Vif on Elongin B were also probed. Deuterium incorporation into Elongin B (Fig. 1d) was not as dramatically affected by Vif as was Elongin C but Elongin B did show a slight decrease in deuterium uptake upon incubation with Vif. This decrease in deuterium content of Elongin B could propagate from the conformational changes in Elongin C, perhaps as a result of changes at the Elongin BC heterodimeric interface. Taken together, these results indicate that upon incubation with full-length recombinant HIV-1 Vif in vitro, the conformation and/or dynamics of both Elongins in the Elongin BC complex is affected.

The HIV-1 Vif:Elongin interaction is specific and requires the C-terminal portion of Vif

Having established that Vif binds to Elongin BC complex in vitro, we next tested the specificity of the interaction. Several different Vif constructs were analyzed (Fig. 2a): wt Vif, Vif1–141, Vif135–158, and VifL145A. These proteins were over-expressed and purified, and their interactions with recombinant Elongin BC complex were tested in pull-down experiments (Fig. 2b and c) and later with HX MS (see below). Vif1–141 is missing the viral BC box (residues 144–158), which has been shown to be essential for Vif interaction.6 As expected, there was a dramatic reduction in Vif1–141 binding to Elongin BC relative to wt Vif binding (Fig. 2b and c). The L145A mutation in Vif was previously shown to reduce viral infectivity,8,12 and in this in vitro pull-down assay, there was a threefold reduction in Elongin BC binding to VifL145A versus wt Vif. We also tested VifSLQ (144SLQ146 to AAA), and it showed a nearly identical result to VifL145A (data not shown). Interestingly, Vif135–158 had the most binding to the Elongin BC complex in this assay.

Fig. 1. Global HX MS analysis of Elongin B and C upon binding HIV-1 Vif. Mass spectra of the +9 charge state of (a) Elongin C in the Elongin BC complex or (b) Elongin C in the Elongin BC complex incubated with a fourfold molar excess of full-length recombinant HIV-1 Vif. The amount of time the complex was incubated in deuterium is indicated (UN, undeuterated). The dotted lines are placed at an arbitrary m/z value as a visual reference. Relative deuterium uptake curves for (c) Elongin C and (d) Elongin B in the absence (continuous line) and presence (dotted line) of fourfold molar excess of full-length recombinant Vif. The error of intact HX MS measurements was ±2 Da. Back exchange has not been corrected for in these experiments (see Materials and Methods).
This region of Vif contains the residues shown by the Vif\textsubscript{139-192}:Elongin BC crystal structure\textsuperscript{12} to form the first helix of the viral SOCS box. The Vif\textsubscript{140-156} crystal structure does not contain any electron density for residues downstream of the first helix. In other SOCS proteins (e.g., SOCS2 and 4), this additional C-terminal sequence forms two more helices.\textsuperscript{3,5} Therefore, the remaining residues of the Vif SOCS box C-terminal to the Viral BC box could interact with the Elongin BC complex via contacts not apparent in the crystal structure and explain the partial binding observed when residues \textsuperscript{144}SLQ\textsuperscript{146} are mutated in full-length Vif. Our results are in agreement with a recent report showing that Vif\textsubscript{139-192} L145A was still able to interact with the Elongin BC complex.\textsuperscript{22} Taken together, our results indicate that deletion (Vif\textsubscript{1-141}) and not mutation (Vif\textsubscript{L145A}) of one or several residues in the viral BC box of Vif abolishes the interaction with Elongin BC \textit{in vitro} and that recombinant Vif and Elongin BC are functionally active and able to interact with one another.

Having established that the Vif:Elongin BC \textit{in vitro} interaction is specific with recombinant proteins, we used HX MS to determine the dissociation constants between the Elongin BC complex and the different Vif constructs. HX MS can probe dissociation constants for protein–protein and protein–ligand interactions that are not easily assayed by other biophysical techniques.\textsuperscript{24} The affinity of full-length HIV-1 Vif with the components of the E3 ubiquitin ligase has not, to our knowledge, been previously reported. The concentration of the Elongin BC complex was held constant while the concentration of wt Vif was varied. Each concentration combination was labeled with deuterium and the mass of all the proteins was measured (as previously described\textsuperscript{24}). As shown in Fig. 3a, it was apparent that as the concentration of Vif increased, more and more protection was afforded to Elongin C. Two populations were apparent in the HX MS data: one that was bound and heavily protected from labeling (Fig. 3a, red dotted line) and one that was unbound and not as protected (Fig. 3a, blue dotted line). With increasing amounts of Vif, the amount of Elongin C in the unbound state (unprotected and heavily deuterated) decreased and the amount of bound Elongin C (protected and less deuterated) increased. The percentage of bound Elongin C was graphed versus Vif concentration (in micromolar) and is shown in Fig. 3b. Consistent with previous observations,\textsuperscript{3} the interaction between Elongin B and C in the Elongin BC complex is very strong (it survives cell lysis and both anion-exchange and gel-filtration chromatography, see Supplementary Fig. S3); therefore, no dissociation between these two proteins was included in the analysis. For the wt Vif:Elongin BC interaction, an equal molar amount of Vif to Elongin BC was required for 100% bound Elongin C, suggesting that the stoichiometry of the wt full-length Vif: Elongin BC interaction was 1:1. The assessment of the binding stoichiometry between HIV-1 Vif and the Elongin BC complex was conducted by fitting the HX MS titration data with a single-site binding model. We note that recently, Bergeron \textit{et al.} showed that the C-terminal tail of Elongin B interacted with Vif and therefore the entire Vif: Elongin BC interaction might not be constituted by...
a single binding site but rather by several regions that involve all three proteins. The HX-MS-derived dissociation constant for the wt Vif: ELC interaction from duplicate HX MS titration experiments was 1.9 ± 0.2 μM. This value is in agreement with a recent ITC report of 1.19 μM for VifΔSLQ whereas it is quite different from that in another report where ITC measurements determined the Kd for VifΔ139−192 to be 0.4 nM. We are unable to explain the large disparity between two of the reports and the third. Although previous studies suggest that Vif145A abolishes the interaction between recombinant Vif and the Elongin BC complex, our results indicate that Vif145A still has the ability to interact with the Elongin BC complex in vitro, albeit with a decreased affinity as compared to wt Vif. VifΔSLQ had a similar affinity as Vif145A (data not shown). Our HX-MS-derived dissociation constant for Vif145A (3.9 μM) is in agreement with that recently reported (4.6 μM) for VifΔ139−192 145A. No binding was detected between Vif1−141 and the Elongin BC complex using our HX MS titration assay, as expected, since Vif1−141 lacks the viral BC box required for interaction with Elongin BC. VifΔ135−158 showed the highest affinity for the Elongin BC complex (0.23 μM), nearly a 10-fold increase in affinity for the Elongin BC complex as compared to wt Vif. The trends observed with HX MS titration were consistent with our pull-down analyses (Fig. 2) and it is clear that recombinant full-length Vif has the ability to interact with Elongin BC in vitro. The additional residues in full-length Vif somehow contribute to decreased Vif binding to the BC complex, perhaps by conformationally restricting the ability of the disordered C-terminal portion of Vif (in the absence of Cullin 5 and Elongin BC16) to adopt a structure capable of binding. The shortened version, VifΔ135−158, with its higher affinity may more easily adopt a conformation competent with binding than the full-length form or perhaps other portions of the full-length form interfere with binding. Regardless of the differences in apparent affinities between Vif proteins and the Elongin BC complex, the ability of Vif to bind the Elongin BC complex in vitro, whereas other cellular SOCS-box-containing proteins require co-expression with Elongin BC, might be a unique property of Vif allowing Vif to escape an additional regulatory mechanism that prevents cellular SOCS proteins from binding with Elongin BC without the aid of other cellular components. This property of Vif ensures that HIV-1 has the ability to hijack the E3 ligase machinery to maintain effective viral replication.

**Localizing conformational flexibility in the Elongin BC complex**

The Elongin C structure in solution has been shown to be dynamic and unstable. The relevance of these findings in the context of the Elongin BC bound to Vif has not been previously reported probably as a result of the high conformational flexibility of Elongin C, the dynamic C-terminal domain of Elongin B, and difficulty in
producing suitable quantities of soluble Vif. We utilized HX MS with pepsin digestion (reviewed in Ref. 20) to localize which regions of Elongin B and Elongin C were dynamic and determine how these regions may change conformational flexibility in the presence of Vif. Simply, pepsin is used to cleave the deuterium-labeled protein into fragments, and the deuterium incorporation in each of the fragments is measured as a function of labeling time. Because the sequence of each fragment can be determined, deuterium incorporation can be localized to each peptic peptide. Note that all deuterium exchange is quenched prior to digestion; hence, the amount of deuterium in each peptide reports on the conformation of the protein under physiological labeling conditions.

Figure 4 summarizes the results of the pepsin digestion experiments. The regions where Elongin C makes contacts with Elongin B in the formation of the Elongin BC heterodimer showed a slow incorporation of deuterium, consistent with a high degree of solvent protection or conformational stability. These regions in Elongin C included the first β sheet (S1), loop 1 (L1), and a section of helix 3 (H3), which were each ≤30% deuterated after 20 min in 2H2O. Residues 29–62 in the N-terminal region of Elongin C, including S2, S3, H1, H2, L2, and L3, were ≥50% deuterated after 20 min in 2H2O. The C-terminal ligand binding domain of Elongin C, including a section H3, L5, and H4, was also heavily deuterated. In contrast, most of Elongin B was quite protected from deuteration,
implying much more conformational stability. Residues 1–85 of Elongin B are part of the N-terminal ubiquitin-like domain of Elongin B, while residues 86–118 constitute the C-terminal tail.\textsuperscript{31} As shown in Fig. 4b, residues 1–62 were \textgreekle 40% deuterated after 20 min in \textsuperscript{2}H\textsubscript{2}O. The C-terminal portion of Elongin B, however, was much more easily deuterated; that is, residues 88–116 become \textgreekge 60% deuterated after 20 min in \textsuperscript{2}H\textsubscript{2}O. These results indicate that the C-terminal portion of Elongin B is quite dynamic, solvent exposed, and not hydrogen bonded in solution and are consistent with the lack of structural data on the C-terminal portion of Elongin B as such a dynamic region would be generally difficult to observe with crystallography.

Mapping of the HX MS data for the Elongin BC complex onto the Elongin BC X-ray structure [Protein Data Bank (PDB): 1LQB]\textsuperscript{30} is shown in Fig. 4c (see also Supplementary Material, Movie S1). The high degree of deuteration in the Elongin C protein when part of the Elongin BC complex suggests that Elongin C is highly flexible in solution and may not be as well ordered as it appears in the crystal. Numerous 3D structural data of Elongin BC bound to SOCS-box-containing proteins have been reported (e.g., Refs. 3, 4, and 12), but to date, there is no published structure of the Elongin BC heterodimer without a SOCS box protein bound. We suggest that binding to a SOCS box protein helps to “lock down” Elongin C and stabilize it such that crystallization and high-quality diffraction can occur; the conformational flexibility of Elongin C in the unbound state, as observed here, would be enough to prevent crystallization. Although there is evidence in the literature\textsuperscript{25–34} that recombinant full-length Vif may exist in a multimeric state, the full-length Vif we have prepared and used for these experiments is still capable of binding to and eliciting an effect on Elongin BC that is completely consistent with the structure of Elongin BC.

**Vif stabilizes Elongin BC**

It has been shown previously with NMR that upon interaction with the VHL SOCS-box-containing peptide, Elongin C undergoes a conformational change.\textsuperscript{18} Elongin BC was mixed with Vif and labeled with deuterium to determine if, how, and where Vif might be able to stabilize the Elongin BC complex upon binding. The location of deuterium incorporation into Elongin BC was then determined following pepsin digestion and MS analysis. Deuterium uptake curves for Elongin BC peptides displaying changes upon incubation with Vif (Fig. 5) show that several regions in Elongin C had marked differences in deuterium in the presence of Vif. These regions included residues 29–46 (S2, H1, L2, and H2), 76–100 (H3 and L5), and 105–109 (H4). Much less deuterium was incorporated into Elongin C in these regions when bound to full-length Vif. Elongin C H3, H4, and L5 make contacts with SOCS-box-containing proteins including Vif\textsuperscript{12} (see Fig. 5b and c), and the changes in deuterium uptake lie within the binding pocket for Vif on the surface of Elongin C. Elongin B residues 63–79 (S5) also displayed a decrease in deuterium uptake upon interaction with Vif. The decreased dynamics of this region of Elongin B upon ligand binding could be explained by its proximity to the ligand binding region of Elongin C (H3, L5, and H4), which becomes stabilized by Vif binding (see Fig. 5c). The decrease in deuterium uptake of S2, H1, and H2 of Elongin C is consistent with the idea that binding of SOCS-box-containing ligands alters the stability and conformation of Elongin C and, in the context of Vif binding, results in conformational changes that localize to the Vif:Elongin C interface.

**Vif’s viral BC box undergoes a structural change in the presence of Elongin BC**

It is unknown if Vif itself undergoes conformational change as a result of binding the Elongin BC complex. Reingewertz et al.\textsuperscript{16} did not detect changes in the C-terminal domain of Vif upon incubation with an Elongin C peptide.\textsuperscript{16} Other folded SOCS box motifs have been observed in numerous structures with the Elongin BC complex,\textsuperscript{1,5} and it was reported that the SOCS box of SOCS3 folded into the helical structure observed with NMR upon complexation with Elongin BC.\textsuperscript{1} If Vif were to gain structure or change conformation in the presence of the Elongin BC complex, it would likely cause a difference in deuterium incorporation. In deuterium exchange experiments at the whole protein level, we were unable to detect major global conformational changes in Vif (peptic peptide level changes in Vif will be reported elsewhere) upon interaction with the Elongin BC complex (see Fig. S5). To further probe this interaction, in terms of potential structural changes in Vif, we utilized Vif\textsubscript{135–158} in an HX MS experiment. HX MS data were obtained for Vif\textsubscript{135–158} in the absence (Fig. 6a) or presence (Fig. 6b) of Elongin BC. Note that after 10 s, the mass increase of Vif\textsubscript{135–158} alone did not change compared to the 1.5-h time point. These data are indicative of an unstructured peptide and suggest that Vif\textsubscript{135–158} does not contain any structural elements in its unbound state. When a calculation\textsuperscript{13} is made for what one would expect to find in a totally unstructured peptide of the same sequence as Vif\textsubscript{135–158} (gray line, Fig. 6c), it closely resembles what is measured for Vif\textsubscript{135–158} in solution (black line, Fig. 6c). The red curve in Fig. 6c is the deuterium uptake for Vif\textsubscript{135–158} upon incubation with the Elongin BC complex based on the data in
Fig. 6b. Note initially that there was a large decrease in deuterium uptake, which became less apparent the longer the complex was labeled in $^2$H$_2$O. This reduction in deuterium uptake is consistent with changes in solvent accessibility and hydrogen bonding in the Vif$_{135-158}$, indicative of formation of structure in the presence of the Elongin BC complex (note that HX MS cannot be used to determine if the structure induced is a helix). While full-length Vif does not appear to change its global conformation upon binding (Fig. S5), the local region that is the main binding element (residues 135–158) does appear to change. A plausible explanation as to why changes are seen in Vif$_{135-158}$ but not in full-length Vif is that other changes in full-length Vif conformation lead to increases in deuterium such that when viewed in total, the decreases in 135–158 are partially cancelled by increases elsewhere. The reduction in deuterium incorporation seen in Vif$_{135-158}$ is too large to be explained merely by solvent protection, thus implying that structural elements have formed in Vif$_{135-158}$. Our results are consistent with known hypotheses indicating that structural elements (i.e., helices) must form in order for the SOCS box proteins to bind to Elongin BC.

Conclusions

Previous studies provide evidence that HIV-1 Vif associates with the Elongin BC complex in vivo and that this interaction is essential for the degradation of APOBEC3 enzymes. Our results show that full-length Vif specifically binds to the Elongin BC complex.
complex in vitro and that this interaction does not require other cellular components. Figure 7 is a summary of the Vif:Elongin BC interaction as revealed by our results. In the unbound state, Elongin C is quite dynamic while Elongin B is quite stable. Upon binding to Vif, with a $K_d$ of $\sim 1.9 \mu M$ for full-length Vif, Elongin C is stabilized as a small region of Elongin B that makes contact on the backside of the Elongin C residues that are stabilized. The Vif BC box folds while the remainder of the C-terminal domain of Vif likely stays disordered. To our knowledge, Vif is the first SOCS-box-containing protein that has the ability to associate with the Elongin BC complex without the aid of other cellular factors or co-expression in vivo. Numerous cellular SOCS-box-containing proteins such as the VHL protein and SOCS1–7 only bind to the Elongin BC complex when co-expressed in vivo.4,38 The formation of the VHL:Elongin BC complex alone, Elongin C is rapidly deuterated, implying that the conformation is very flexible. Elongin B, in contrast, is much more difficult to deuterate, implying that it is more stable in solution. Unlike other SOCS-box-containing proteins,15 recombinant HIV-1 Vif interacts with the recombinant Elongin BC complex in vitro without additional factors. Full-length HIV-1 Vif (wt) binds to the Elongin BC complex with a $K_d$ of $\sim 1.9 \mu M$ and stabilizes Elongin C and a small region in Elongin B. The main region in Vif (the BC box residues 135–158) that is responsible for interacting with Elongin C folds upon binding.

Fig. 6. The Vif BC box becomes structured upon binding to Elongin BC. Deuteration of Vif$_{135-158}$ in (a) the absence of the Elongin BC complex or (b) the presence of the Elongin BC complex. The +5 charge state of Vif$_{135-158}$ is shown. The deuterium labeling time is shown at the right and applies to spectra in both (a) and (b) (UN, undeuterated). The dotted lines are provided for visual guidance. (c) Deuterium uptake curves for data shown in (a) and (b). The gray dotted line is the theoretical deuterium uptake for an unstructured peptide of the same sequence as calculated according to the Bai/Molday factors.35 The theoretical deuterium uptake was adjusted by subtracting the amide positions of the 6×His tag due to the fast-exchange properties of this sequence (i.e., all His residues should be undeuterated in Vif$_{135-158}$ under the conditions used).36 The data for Vif$_{135-158}$ have been corrected for back exchange (see Materials and Methods). Note that the exchange properties of Vif$_{135-158}$ in the absence of the Elongin BC complex (black line) nearly overlay the theoretical deuterium uptake for the Vif$_{135-158}$ sequence if it did not contain any higher-order structure (dotted line). Upon incubation with the Elongin BC complex (red line), Vif$_{135-158}$ undergoes changes in deuterium uptake, which are indicative of changes in solvent accessibility and hydrogen bonding.37 The y-axis maximum is set to the number of backbone amide hydrogens in Vif$_{135-158}$ capable of exchanging.

Fig. 7. Summary of the behavior of Elongin BC based on our results. In the Elongin BC complex alone, Elongin C is rapidly deuterated, implying that the conformation is very flexible. Elongin B, in contrast, is much more difficult to deuterate, implying that it is more stable in solution. Unlike other SOCS-box-containing proteins,15 recombinant HIV-1 Vif interacts with the recombinant Elongin BC complex in vitro without additional factors. Full-length HIV-1 Vif (wt) binds to the Elongin BC complex with a $K_d$ of $\sim 1.9 \mu M$ and stabilizes Elongin C and a small region in Elongin B. The main region in Vif (the BC box residues 135–158) that is responsible for interacting with Elongin C folds upon binding.
complex requires the chaperonin TRiC and SOCS1–7 requires co-expression with Elongin BC to form a functional complex. The ability of Vif to specifically interact with the Elongin BC complex independent of other cellular factors might be a unique property of Vif that allows it to more easily recruit components of the E3 ligase machinery.

We were unable to detect global conformational changes in full-length Vif upon incubation with Elongin BC. Deuterium incorporation into a Vif135–158 peptide changed dramatically upon Elongin BC incubation and suggests that this region folds (into a helical structure according to similarity with other SOCS systems) upon binding and likely does so in the full-length protein. Such an event would be consistent with the structures of SOCS-box-containing proteins seen in numerous structures of the Elongin BC complex, including that observed for SOCS3. The physiological relevance of the low micromolar dissociation constants reported in this study and that of Bergeron et al. can likely be attributed to several factors. Additional stabilization of the Vif:Elongin BC complex could possibly arise from cellular factors present in vitro that were absent in vitro such as Cullin 5 and/or zinc. The dynamic and unstructured nature of the C-terminal tail of Vif could also explain the moderate affinity of full-length Vif, compared to shorter Vif constructs, for Elongin BC. The entropic costs of folding would be less significant in shorter Vif constructs and the possible steric effects of the additional residues present in full-length Vif would be greater than that in Vif135–158. The necessity of a high-affinity interaction between Vif and the Elongin BC complex might be partially negated due to the additional mechanisms in which Vif circumvents the antiviral activities of the APOBEC3 proteins seen in numerous structures of the Vif:Elongin BC complex.

Pull-down analysis of the HIV-1 Vif:Elongin BC Interaction

For pull-down experiments between Vif constructs and the Elongin BC complex, 1 nmol of each Vif construct was immobilized on Ni-NTA agarose. The Vif-immobilized Ni-NTA agarose was then incubated with 3 nmol of the Elongin BC complex for 30 min at 4 °C. After incubation, each reaction was washed 7 times with 1 mL each of Vif buffer. Twenty microfilters of each reaction were then analyzed with SDS-PAGE and visualized with Coomassie Blue. Twenty microliters of each reaction was then injected into a Shimadzu SCL-10A VP HPLC flowing at 200 μL/min coupled to a Waters LCT premier mass spectrometer. The Vif after dilution into 2H2O were 0.36 and 1.45 μM. The labeling reaction was quenched by adjusting the pH to 2.6 with 0.8 M guanidine hydrochloride and 80 °C. All protein masses were verified by MS and are shown in the Supplementary Fig. S1.

DNA constructs and protein purification

Full-length HIV-1 Vif (HXB2), Vif145A, VifSLQ (SLQ to AAA), and Vif1–141 were over-expressed and purified using the pET28b vector as previously described. The HIV-1 Vif pET28b codon-optimized construct was a gift from Dana Gabuzda at Harvard Medical School. VifL145A, ΔSLQ, and 1–141 were constructed using Lightning QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis from the HIV-1 Vif (HXB2) template. Human Elongin B and C (residues 17–122) were co-expressed using the pACYCDUET-EICB vector in BL21(DE3). The pACYCDUET-EICB vector was a gift from Alex Bullock and Stefan Knapp at the Structural Genomics Consortium, University of Oxford. The Elongin BC complex was purified by anion exchange on a HiTrap QHP column followed by size-exclusion chromatography on a Superose 12 10/300 column (GE Healthcare). All purified proteins were dialyzed into Vif buffer (20 mM Mops, 150 mM NaCl, 1 mM DTT, and 10% glycerol, pH 7.0) and stored at −80 °C. Vif135–158 (HHHHHHH135QYAGHNVGSLQYLAAL158) was designed from the HXB2 Vif viral BC box sequence with a 6×His affinity tag and synthesized by GenScript (Piscataway, NJ). The peptide (>98% purity) was dissolved directly into Vif buffer and stored at −80 °C. All concentration values in all experiments were determined with the Bradford assay (Bio-Rad protein assay, Hercules, CA). All protein masses were verified by MS and are shown in the Supplementary Fig. S1.
PerSeptive Biosystems), and all associated tubing were kept at 0 °C to minimize back exchange.20 Deuterium levels were not corrected for back exchange and are therefore reported as relative deuterium levels.20 Intact mass spectra were deconvoluted using the software McCy, which was applied (except for that noted in the next paragraph) and all values are therefore reported as relative.20 All experiments were conducted at least twice. For experiments with the Elongin BC complex and Vif, the deuterium-labeled protein.

HX MS titrations

For Elongin BC titrations with HIV-1 Vif, each Vif construct was titrated into a fixed concentration of Elongin BC (3.22 μM). The Vif-Elongin BC mixtures were incubated at 0 °C for 10 min before labeling. The mixtures were then diluted with a 10-fold excess of 20 mM Mops, 150 mM NaCl, 1 mM DTT (pD 7.0), 2H2O at 20 °C and labeling was allowed to proceed for 10 s before quenching. After the quench, protein mixtures were analyzed as stated above. The percentage of Elongin C bound and unbound was determined by fitting the area under a Gaussian distribution to the fast-exchange properties of this sequence.36

Peptide-level HX MS

HX was performed with a 10-fold dilution into 2H2O and then quenched as stated above. Quenched samples (110 μL total volume, 20 pmol of Elongin BC) were digested, desalted, and separated online using a custom Waters nanoACUITY UPLC system.44 Online digestion was desalted, and separated online using a custom Waters nanoACUITY UPLC system.44 Online digestion was performed in 0.05% formic acid at 20 °C at a flow rate of 50 μL/min through a 2.1 mm × 50 mm stainless steel column packed with pepsin immobilized on POROS-20AL beads (PerSeptive Biosystems). Peptidic peptides were trapped on an AQUITY UPLC BEH C8 1.7 μm 1.0 × 100 mm column (Waters, Milford, MA) at 0 °C with a flow rate of 40 μL/min. A 6-min 0–40% acetonitrile gradient with 0.05% formic acid (pH 2.6) was used to elute the peptides directly into a Waters QToF premier mass spectrometer with standard electrospray interface. Continuous low mass correction was performed using Glu-fibrinogen peptide, and peptidic peptides were identified using MS² and Waters Identity software.46 The deuterium uptake for each peptide was determined using the Excel-based software program HX-Express by subtracting the centroid mass of each undeuterated peptide from the centroid mass of deuterium-labeled peptides. No back exchange correction was applied (except for that noted in the next paragraph) and all values are therefore reported as relative.20 All experiments were conducted at least twice.

For experiments with the Elongin BC complex and Vif, Vif (4.00 μM) was incubated with or without a fourfold molar excess of the Elongin BC complex (16 μM). The percentage of Vif/Vif, Vif, Vif/Vif, Vif/135–158 bound to the Elongin BC complex during equilibration was calculated to be 83% based on a Kd of 0.23 μM. Deuterium uptake values for Vif/Vif, Vif, Vif/Vif, Vif/135–158 were adjusted for back exchange in order to make a comparison with the predicted theoretical exchange. For the fully deuterated control, Vif/Vif, Vif, Vif/135–158 was incubated at 37 °C for 7 h in a 10-fold excess of 2H2O before quenching and analysis as above. For back exchange correction, Eq. (1) was used.

\[ D_0 = \frac{(m) - (m_{2H})}{(m_{100}) - (m_{2H})} \times N \]

D0 is the back-exchange-adjusted amount of deuterium atoms incorporated into a protein after incubation in 2H2O. (m), (m2H), and (m100%) are the observed masses of the protein partially deuterated, undeuterated, and fully deuterated, respectively. N is the number of exchangeable amide positions in a protein. N was adjusted for by subtracting the amide positions of the 6×His tag due to the fast-exchange properties of this sequence.

Supplementary material related to this article can be found online at doi:10.1016/j.jmb.2010.08.026.

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References


SUPPLEMENTARY MATERIAL

Figure S1.
Mass spectra of proteins used for conformational studies. (A-E,i) Intact mass spectra of Elongin BC and Vif constructs used. (A-E,ii) Deconvoluted mass spectra of Elongin BC and Vif constructs used.

Figure S2.
HX MS titration results for all Vif:Elongin BC interactions. Panel A is the same as the data shown in Figure 3. The +7 charge state of Elongin C is shown in the presence of each Vif construct at the ratios indicated. As in Figure 3, the blue distribution represents the unbound form of Elongin C and the red distribution represents the bound form.

Figure S3.
SDS PAGE gels of (A) anion exchange and (B) size exclusion chromatography purification steps of the Elongin BC complex. The two proteins have high affinity for one another and are always found together from the moment of expression.

Figure S4.
HX MS peptide data for Elongin B and C. These data include those shown in Figure 5. All peptides that could be followed are shown. Some peptides do not have data in the presence of Vif due to interference from Vif peptic peptides.
Figure S5.
Deuterium exchange into intact Vif in the absence and presence of the Elongin BC complex.
The error of determining the deuterium level was ± 2 Da.

Movie S1.
Animation (approximately 6 seconds) illustrating the deuterium incorporation for the pepsin
digested Elongin BC over time. Frames are: 0, 10 s, 1 m, 5 m, and 20 m, as indicated. See also
Figure 4C.
A. i
Elongin BC

Elongin BC
Theoretical 10963.6 Da
Observed 10962.9 Da

B. i
Vif\textsubscript{135-158}

Vif\textsubscript{135-158}
Theoretical 3407.86 Da
Observed 3406.9 Da

C. i
wt Vif

Vif\textsubscript{145A}
Theoretical 22512.9 Da
Observed 22512.1 Da

D. i
Vif\textsubscript{141}

Vif\textsubscript{141}
Theoretical 16757.1 Da
Observed 16756.5 Da

E. i
Vif\textsubscript{145A}

Vif\textsubscript{145A}
Theoretical 22470.8 Da
Observed 22470.5 Da
Figure S2
A. Elongin BC anion exchange fractions

B. Elongin BC size exclusion fractions

Figure S3
Elongin C peptic peptides

\[ \text{VLI} \text{SSDGKE}^{28} \]

\[ \text{REPSHVLKKWC}^{75} \]

\[ \text{FOV]KREDKALTSGTKAML}^{46} \]

\[ \text{FTKVNTSPEPPEA}^{96} \]

\[ \text{FTYKV} \text{R]NTSPEPPEA}^{100} \]

\[ \text{MA} \text{NN}^{109} \]

\[ \text{SSPQGFE}^{52} \]

\[ \text{ENETNEV}^{52} \]

Elongin B peptic peptides

\[ \text{WKQLD} \text{GKLGE}^{62} \]

\[ \text{TSQRTAPDA} \text{FATVG}^{79} \]

\[ \text{LMR[} \text{KTTIFT}^{16} \]

\[ \text{DAKE} \text{S}^{25} \]

\[ \text{CEPFS} \text{SPPELPOMQPGDSSANEGA}^{116} \]

\[ \text{LRKP}^{64} \]

\[ \text{L}^{32} \]

\[ \text{AFRADD}^{85} \]

\[ \text{MD]V}^{5} \]

\[ \text{GL} \text{KRPDC}^{61} \]

Figure S4
Figure S5

Figure showing the relative deuterium level (Da) over time (min) with max = 181. The graph compares unbound and bound states with time increasing from 0.1 to 100 minutes.