Title
Downregulation of MHC-I by HIV-1 Nef: evolution after sexual transmission and mechanism of action

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Downregulation of MHC-I by HIV-1 Nef: Evolution after Sexual Transmission and Mechanism of Action

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Colleen M. Noviello

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2007
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in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007
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<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>AIEDRP</td>
<td>Acute Infection and Early Disease Research Program</td>
</tr>
<tr>
<td>AP-1</td>
<td>adaptor protein complex 1</td>
</tr>
<tr>
<td>APOBEC3G</td>
<td>Apolipoediting protein B</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC-type chemokine receptor 5</td>
</tr>
<tr>
<td>CD</td>
<td>cytoplasmic domain</td>
</tr>
<tr>
<td>CD-MPR</td>
<td>cation dependent-mannose 6-phosphate receptor</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CI-MPR</td>
<td>cation independent-mannose 6-phosphate receptor</td>
</tr>
<tr>
<td>CK II</td>
<td>casein kinase II</td>
</tr>
<tr>
<td>COP</td>
<td>coat protein</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC-type chemokine receptor 4</td>
</tr>
<tr>
<td>Env</td>
<td>envelope</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>gag</td>
<td>Group antigen protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanidine tri-phosphate</td>
</tr>
<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma herpesvirus</td>
</tr>
<tr>
<td>MHC-I</td>
<td>class I major histocompatibility complex</td>
</tr>
<tr>
<td>MHC-II</td>
<td>class II major histocompatibility complex</td>
</tr>
<tr>
<td>MVB</td>
<td>multi-vesicular body</td>
</tr>
<tr>
<td>Nef</td>
<td>negative factor</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>PACS-1</td>
<td>phosphofurn acidic cluster sorting protein-1</td>
</tr>
<tr>
<td>PIC</td>
<td>pre-integration complex</td>
</tr>
<tr>
<td>pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>PR</td>
<td>protease</td>
</tr>
<tr>
<td>REL</td>
<td>random effects likelihood</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SIV</td>
<td>simian immunodeficiency virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter of antigenic peptides</td>
</tr>
<tr>
<td>Tat</td>
<td>trans-activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>TGN38</td>
<td>trans-Golgi network resident protein 38kD</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity protein</td>
</tr>
<tr>
<td>Vpr</td>
<td>viral protein r</td>
</tr>
<tr>
<td>Vpu</td>
<td>viral protein u</td>
</tr>
<tr>
<td>vRNA</td>
<td>viral RNA</td>
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**Amino Acid Residues**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Amino Acid</th>
</tr>
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<tbody>
<tr>
<td>Ala or A</td>
<td>alanine</td>
</tr>
<tr>
<td>Arg or R</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn or N</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp or D</td>
<td>aspartate</td>
</tr>
<tr>
<td>Cys or C</td>
<td>cysteine</td>
</tr>
<tr>
<td>Glu or E</td>
<td>glutamate</td>
</tr>
<tr>
<td>Gln or Q</td>
<td>glutamine</td>
</tr>
<tr>
<td>Gly or G</td>
<td>glycine</td>
</tr>
<tr>
<td>His or H</td>
<td>histidine</td>
</tr>
<tr>
<td>Ile or I</td>
<td>isoleucine</td>
</tr>
<tr>
<td>Lys or K</td>
<td>lysine</td>
</tr>
<tr>
<td>Leu or L</td>
<td>leucine</td>
</tr>
<tr>
<td>Met or M</td>
<td>methionine</td>
</tr>
<tr>
<td>Phe or F</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>Pro or P</td>
<td>proline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Ser or S</td>
<td>serine</td>
</tr>
<tr>
<td>Thr or T</td>
<td>threonine</td>
</tr>
<tr>
<td>Trp or W</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Tyr or Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Val or V</td>
<td>valine</td>
</tr>
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</table>
ACKNOWLEDGMENTS

“A doctorate is a marathon, not a sprint.” I thank my father, for amongst a million other words of encouragement and support, these were some that came back to me again and again during the long years when nothing worked. A crucial, if not the most important, part of a doctorate is the mentor. With a less patient, brilliant, or kind mentor than John Guatelli, who knows where I would be now. John took on a headstrong, hard working, talking-back student and turned me into a scientist. I thank him for always listening to my outlandish ideas and conspiracy theories, even when I interrupted his train of thought to say them. John’s intelligence is formidable, but he never held it over me (or anyone); a humble personality is not usually associated with brilliance, but in John Guatelli’s case, he had both. I’m so lucky to have worked with him, and I’ll miss it—thanks John!

I am so grateful to my mother, who, in my 28 years never stopped expressing her love, pride and support for me. My sister, who inspires me whenever I think about her, makes me laugh harder than anybody else, and understands me without having to say a lot. I also want to thank my therapist, Brenda Johnson. I struggled with depression for years, and all my family and friends had to deal with me like that; I am so grateful to her for helping me through it and to become a balanced person. My friends, especially Ramlah, Jen, Shannon, Scarlett, James and Camilla, got me through it all.

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Chapter IV, in part, has been submitted for publication to the Journal of Biological Chemistry as: “Cooperative binding of the MHC-I cytoplasmic domain and HIV-1 Nef to the endosomal AP-1 complex,” Colleen M. Noviello, Serge Benichou, and John C. Guatelli. The dissertation author was the primary investigator and author of these papers.
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Major Field: Biomedical Sciences

Studies in Viral and Cellular Protein Interactions
Professor John C. Guatelli
PUBLICATIONS


ABSTRACTS

Noviello CM, Benichou S and Guatelli JC. Binding of the MHC-I cytoplasmic domain/Nef complex to the endosomal sorting machinery: leucine-independent, tyrosine-based interaction with the μ subunit of AP-1. 2007 Keystone Symposia on the Molecular and Cellular Determinants of HIV Pathogenesis.


ABSTRACT OF THE DISSERTATION

Downregulation of MHC-I by HIV-1 Nef: Evolution after Sexual Transmission and Mechanism of Action

by

Colleen M. Noviello

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2007

Professor John C. Guatelli, Chair

The Human Immunodeficiency Virus (HIV) Nef protein has evolved multiple methods to evade the host immune system. Reduction in cell-surface major histocompatibility complex I (MHC-I) is one such method; this enables virally-infected cells to evade lysis by cytotoxic T lymphocytes (CTL). In addition, Nef also performs functions related to HIV infectivity and replication. The main objective of this dissertation is to delineate the mechanism by which Nef downregulates MHC-I, and how this function may be sacrificed in order to optimize infectivity and replication.
I first focused on the optimization of Nef functions after sexual transmission by studying the modulation of MHC-I and CD4 by Nef clones from phylogenetically- and epidemiologically-linked transmission partners. Flow cytometry of T cells transfected with these Nef clones showed that both CD4 and MHC-I downregulation are important to the establishment of a successful infection in a new host and neither function is expendible.

Next I addressed how Nef modulates MHC-I trafficking in the cell by examining the interactions of Nef, MHC-I, and the non-clathrin endocytic pathway. I studied the role of the GTP cycle of the ADP-ribosylation factor 6 (ARF6) in the Nef-mediated downregulation of MHC-I. Immunofluorescence microscopy of cells transfected with both Nef and epitope-labeled ARF6 were examined for colocalization. Additionally, T cells transfected with Nef, and mutants of ARF6 that interfered with the ARF6-GTP cycle, were examined for cell-surface MHC-I by flow cytometry. While disruptions of the ARF6-GTP cycle did appear to interfere with MHC-I cell-surface expression, this result was not related to MHC-I downregulation by Nef.

Finally, I changed the focus of the mechanistic studies to the intracellular endosomal system, specifically to adaptor protein-1 (AP-1)-containing vesicles. Studying protein-protein interactions, I found that Nef and MHC-I can synergize to create a novel AP-1 interaction surface, and I determined residues in Nef, MHC-I, and the subunit of AP-1 involved in this interaction.

In summary, my dissertation research has contributed to a greater understanding of not only the importance of MHC-I downregulation by HIV-1 Nef, but also to its mechanism in the cell.
Chapter I: Introduction to the Human Immunodeficiency Virus and the Nef protein
General Overview of HIV-1 History, Transmission and Disease

Acquired Immunodeficiency Syndrome (AIDS) was first recognized in 1981, described as the rare occurrence of *Pneumocystis carinii* in five homosexual men in Los Angeles (3). Shortly thereafter, the causative agent was isolated from blood by the laboratories of Luc Montagnier at the Pasteur Institute (Paris, France) and Robert Gallo at the National Cancer Institute (Bethesda, Maryland). It was later given the name Human Immunodeficiency Virus (HIV). Since 1981, over 25 million people have died of AIDS, with the current population of HIV+ individuals estimated to be 40 million worldwide (4). There is no cure for this genetically divergent retrovirus; while medication is effective in stopping the course of the disease, to date there is no option for prevention of infection or eradication of HIV from infected individuals.

HIV and its related simian cousins, the simian immunodeficiency viruses (SIVxx, where xx denotes the host species), are primate lentiviruses. The lenti-stem of lentivirus refers to the fact that these viruses are “slow” at causing disease in their hosts; indeed, in the natural host species for SIV, monkeys usually never progress to AIDS. The origin of HIV is thought to have occurred by zoonotic transmissions of SIVcpz (for HIV-1) and SIVsm (for HIV-2) to humans around 1931 +/-15 years (16,17).

Primary HIV infection results in an acute syndrome that includes symptoms similar to mononucleosis, including fatigue, lymphadenopathy and fever (1). Within 3-6 weeks, the viral RNA copies per milliliter blood (vRNA/ml) reach a peak level, decreasing with the onset of the adaptive immune response (Figure I.1). Eventually, viral levels within the blood reach equilibrium (the “viral set-point”) for 1-10 years, referred to as clinical latency. Without drug treatment, continued viral replication eventually exhausts
the immune system, and as the level of CD4+ T cells per milliliter blood drops below 200, the clinical diagnosis of AIDS is assigned. At this point, the body no longer has an effective cell-mediated defense against such opportunistic pathogens as tuberculosis, candida, and *Pneumocystis jiroveci*, and the individual succumbs to infections that an otherwise healthy person would be able to overcome.

The major obstacle to a strategy for prevention, e.g. the development of a vaccine, is the genetic diversity of HIV that is a product of several factors. As a retrovirus, the replication cycle of HIV is dependent upon an error-prone nucleotide polymerase, reverse transcriptase, which introduces diversity directly through introduced mutations in the viral genome (1). Additionally, two copies of the viral RNA (vRNA) genome are encapsidated in each virion, enabling recombination to occur between these genomes within the viral particle (6). Within the virion and the cell, a host protein, APOBEC3G, acts as a cytidine deaminase, resulting in an increase in the rate of G to A mutations. Finally, a target cell may be infected with more than one virus, and these viruses may not have identical genomes. Thus, within a cell, multiple combinations of the parental genomes may be assembled into daughter virions. All of these functions contribute to the genetic diversity of HIV, giving it an ability to outrun the host immune response by evolving at a rapid rate.

**Genomic organization of HIV and replication cycle within the cell**

After transmission of virus to the new host the first step during HIV infection is entry of the virus to target cells. HIV cannot infect all cells; it requires cells to express at least two specific proteins on their cell surface in order for successful entry. The first, CD4, is considered the primary receptor of HIV, and is only found on a subset of cells
including macrophages and helper T cells. The second is one of a family of receptors normally utilized by the chemical signaling system of the immune system; two of these chemokine receptors, CCR5 and CXCR4, are the main co-receptors of HIV.

After entry into the cell, the viral core is dissembled, or uncoated via unclear mechanisms, and the reverse transcriptase (RT) synthesizes proviral DNA from the vRNA genome (Figure I.2, top). The DNA provirus, along with the matrix, integrase and Vpr proteins form the pre-integration complex (PIC, for review, see (30)). The PIC is trafficked to the nucleus of the host cell, possibly along microtubules (12,23) where it is then inserted into the host DNA via the integrase protein. From this integrated proviral genome, RNA transcripts are synthesized by host-encoded RNA polymerases. Early viral transcripts are multiply spliced and encode nef, tat, and rev (13). Tat is responsible for activating transcription of the HIV provirus, Rev allows export of unspliced viral RNAs from the nucleus, and the functions of the Nef protein will be discussed extensively in the next section of this chapter.

Later transcripts encode proteins from the gag, pol and env genes. The products of gag transcripts are cleaved into the structural proteins capsid, matrix, and nucleocapsid proteins (Figure I.2, bottom panel). The env gene produces the envelope glycoproteins gp120 and gp41, which are expressed as trimers on the surface of virions (for review, see (25)). Subsequently, two copies of the viral RNA genome, exported from the nucleus by Rev and guided by the nucleocapsid protein, assemble with the structural Gag and envelope proteins into immature virions. The assembly of virions occurs within two locations intracellularly. At the plasma membrane, virions bud directly into the extracellular matrix.
Figure I.1. HIV Viral RNA levels vs. CD4+ T cells. During the course of an HIV infection, the level of viral RNA in the blood peaks within the first few weeks, then with the onset of the adaptive immune response (represented as an increase in CD4+ T cells). After this, viral RNA levels reach a “set-point”, an equilibrium which may be maintained until failure of the immune system, at which point the individual is stated to have AIDS (CD4+ T cells <200 cells/ml.) Figure is adapted from aids.quickseek.com
Alternatively, virions have been proposed to bud in multivesicular bodies (MVB), which contain late endosomal components. Later, the entire MVB is thought to fuse to the peripheral membrane, releasing the contained viral particles (24). Maturation of virions occurs when the viral protease (PR) cleaves the Gag polyprotein precursor to form the capsid and matrix proteins (Figure I.2, bottom panel). It is at this point the virus becomes infectious and is capable of infecting the next target cell.

While the accessory proteins of HIV, including Vif, Vpr, Vpu, Tat, and Nef (Figure I.2), are not absolutely required for viral replication they nonetheless play important roles during HIV infection (For review, see (21)). The Vif protein is involved in degrading a host cytidine deaminase, APOBEC3G, that attacks the viral genome (31). The Vpr protein affects the progression of the host cell cycle, induces apoptosis, and is involved in importing the PIC into the nucleus. The small (9kD) Vpu protein functions to enable virion budding from the peripheral membrane, as well as sequestering and degrading CD4 in the endoplasmic reticulum (ER). As mentioned previously, Tat is involved in inducing vRNA transcription from the integrated HIV genome, and Rev exports newly synthesized vRNA by binding to Rev-response elements encoded in the RNA. The functions of Nef will be discussed in detail below.

**Nef functions during HIV infection**

The Nef protein is unique to primate lentiviruses, and plays an important role in infection and disease progression. These functions of Nef may be generally grouped into two categories: effects on intracellular protein trafficking, and effects on signal transduction pathways. The effects of Nef on signal transduction pathways lower the
Figure I.2. The genomic and structural organization of HIV-1. MA, matrix, CA, capsid, NC, nucleocapsid, PR, protease, RT, reverse transcriptase, IN, integrase, env, envelope. Adapted from Frankel and Young, Annual Reviews of Biochemistry 1998; 67:1-25. Reprinted, with permission, from the Annual Review of Biochemistry, Volume 67 © 1998 by Annual Reviews www.annualreviews.org.
threshold for T-cell activation, which theoretically enables target cells to become better viral producers. By modulating the trafficking pathways of target proteins, Nef is able to evade the immune system by downregulating MHC-I and enhance infectivity, both by downregulating CD4 and an unknown, CD4-independent mechanism.

HIV-1 Nef is a small protein, ranging from 27-31 kD. As Nef varies considerably in vivo, for simplicity’s sake all numbering of amino acids henceforth will refer to the reference sequence NL4-3, a chimeric molecular clone derived from two clade B strains (2). Nef is myristoylated on the second residue of its N-terminus, and through this modification is transiently associated with intracellular membranes. The protein structure of Nef can be divided into three domains (Figure I.3). The N-terminal region, which contains the myristoylation signal, is mostly disorded in structure with the exception of an α-helix (residues 16-22), which is required for MHC-I downregulation. The core, spanning from residues 57-142, includes two important regions. The first, a group of acidic residues (EEEE_{62-65}) thought to be involved in interaction with the phosphofurin-associated cell sorting protein-1 (PACS-1), is required for MHC-I downregulation. The second region of the core includes the polyproline helix (P_{69-P_{72}}-P_{75-P_{78}}). The middle two prolines in the helix have been implicated as an SH3-binding domain required for effects on signal transduction, although the SH3-conforming cellular partner is unclear. The last proline has been described as critical to MHC-I downregulation (5,34). Finally, the C-terminus (residues 143-207) constitutes the third domain of Nef. This region
includes an unstructured, flexible loop (residues 143-181) containing an acidic di-leucine motif (E_{160}xxxLL_{165}) necessary for Nef’s effects on viral infectivity and some of its effects on intracellular trafficking (for review, see (8,14))

**MHC-I downregulation by HIV-1 Nef and other viral proteins**

Immune evasion of viral infections is crucial to the success of the pathogen. This biological arms race has caused pathogens to develop numerous mechanisms to thwart the immune system, including inhibition of protein synthesis, increasing protein degradation, and blocking protein-protein interactions required for stimulating an immune response. A common target of these responses is the cell-surface MHC-I, which presents antigenic peptides to CD8+ CTL via MHC-I/T-cell receptor (TCR) interactions. The normal pathway of antigenic presentation by MHC-I, illustrated in figure I.4, is as follows: 1. Intracellular proteins are cleaved by the ubiquitin proteasomal system into small peptides. 2. These peptides are escorted into the ER by a protein known as transporter of antigenic peptides (TAP), where they encounter unassembled MHC-I heavy and light chains. 3. Assembly of the MHC-I complex occurs in the ER, and the complex traffics through the Golgi and *trans*-Golgi apparati. 4. From the *trans*-Golgi, MHC-I is exported to the cell surface, where it presents the antigenic peptide to surveillance CTL. CTL then kill the infected cell, limiting production of the pathogen.

Once on the surface, the complex remains until bulk-phase endocytosis internalizes MHC-I and sends it to a tubular, non-clathrin recycling compartment. Because MHC-I does not contain a canonical sorting signal in its cytoplasmic tail, it is not rapidly internalized via clathrin-mediated endocytosis. However, directed by a tyrosine in its cytoplasmic tail (Y_{320}), some MHC-I can reach a clathrin-coated
intracellular compartment, the so-called “MHC-II compartment”. Here it engages in cross-presentation of extracellular-derived peptides normally associated with presentation by MHC-II (22). MHC-I is then recycled back to the cell-surface, where it can prime naïve CD8+ CTL.

Several viruses inhibit antigen presentation by MHC-I. For example, in human cytomegalovirus (HCMV), six proteins have been implicated in interfering with MHC-I presentation, functioning to induce proteasomal degradation (US2, US11), prevent maturation of the MHC-I heavy chain (US3), prevent TAP from delivering peptides to MHC-I in the ER (US6), and retaining MHC-I within the ER (US3 and US10). Also, human papilloma virus (HPV), Kaposi sarcoma herpesvirus (KSHV) and herpes simplex virus (HSV) all reduce the amount of cell surface MHC-I (for review, see (29)).

This dissertation is focused upon how an HIV protein, Nef, aids in evading the immune response by altering the intracellular trafficking of the MHC-I molecule to reduce the density of viral antigens presented on the surface of infected cells. The mechanism by which Nef directly affects MHC-I trafficking is unique in the viral world, as Nef affects neither MHC-I synthesis nor inhibits the TAP/MHC-I interaction. Instead, it is thought that Nef prevents MHC-I from reaching the cell surface after processing in the ER and Golgi apparatus, as well as by increasing the endocytosis of MHC-I already on the cell surface (19,28). Nef achieves this by directly interacting with the cytoplasmic tail of MHC-I-A and -B proteins (33). It does not affect MHC-I-C proteins, as they lack a crucial tyrosine in the cytoplasmic tail (the same Y_{320} described above), or MHC-I-E protein, as the tyrosine present on MHC-I-E may be in a context unsuitable for Nef interaction (YSQAA in MHC-I-A and –B vs. YKQEW in MHC-I-E). By leaving MHC-I-
Figure I.4 Intracellular trafficking of MHC-I and MHC-II. ER, endosomal reticulum. TGN, trans-Golgi network. TAP, transporter of antigenic peptides.
C and -E on the cell surface, Nef prevents killing by natural killer cells (NK), but evades the CTL-mediated response by removing MHC-I-A and -B (7).

Nef connects the MHC-I molecule to an intracellular sorting pathway away from the cell surface to lysosomes, where it is degraded (27). While a small proportion of MHC-I does reach the cell surface, Nef also induces endocytosis of these molecules. In live cells expressing Nef-GFP and labeled with an antibody against MHC-I, uptake of the labeled MHC-I showed a distribution of MHC-I in the juxtanuclear area (Figure I.4). Cellular components of the sorting pathway that have been implicated in the mechanism of Nef-mediated downregulation of MHC-I include PACS-1 (26), and the adaptor protein complex 1 (AP-1) (27). PACS-1 normally functions to retrieve proteins from the endosomal system to the trans-Golgi network via interactions with acidic cluster regions in the cytoplasmic tail of its cargo. Cells engineered to express reduced amounts of PACS-1 are unable to support Nef-mediated downregulation of MHC-I (26), and PACS-1 has also been implicated in linking Nef and the AP-1 complex via its interactions with the small (σ1) and medium (μ1) subunits of AP-1 (11). However, several studies have shown that Nef is capable of interacting with the hemi-complexes of AP-1 (γ−σ1) and AP-3 (δ−σ3) directly through its di-leucine motif (18). Paradoxically, this ExxxLL motif in Nef is not required for Nef to modulate MHC-I. Perhaps more relevant to the downregulation of MHC-I by Nef is its interaction with μ1, which is independent of the Nef acidic di-leucine motif (10,20).
Figure I.4 The localization of Nef and MHC-I overlaps in a juxtanuclear region. Cells transfected with Nef-GFP were labeled with the W6.32 antibody, which stains MHC-I-A,-B, and -C. Cells were then incubated for four hours at 37°C/5% CO₂ to allow internalization of the antibody from the cell surface and subsequently stained with an antibody labeled with RhodamineX. Yellow puncta in the Merge frame indicate co-localization.
As mentioned previously, regions of Nef known to be involved in reduction of cell-surface MHC-I include the acidic cluster (EEEE<sub>62-65</sub>) and the N-terminal α-helix (16-22), as well as the last proline in the polyproline region (P<sub>78</sub>). The roles of the polyproline helix in MHC-I downregulation is controversial (15). It is important to note that P<sub>78</sub> does not have any role in signal transduction effects, but does affect MHC-I downregulation, separating it from the other prolines in that region. These regions will be examined extensively for their roles in binding to cellular partners of Nef in an attempt to correlate protein interactions with phenotype.

**Endosomal trafficking within the cell**

 Trafficking of cellular proteins within the cell is mediated by a consortium of coat, adaptor, and motor proteins. The external layer of the vesicle can be “uncoated” or “coated” with proteins. The uncoated vesicles can originate from the cell surface, as well as from some internal recycling compartments. Coat components include those of the coat protein (COP) family, which are localized to the Golgi apparatus. Another type of vesicle coat is the clathrin protein, which coats vesicles throughout the endosomal system.

 The adaptor protein complexes are components of the clathrin vesicular trafficking system. There are four known complexes in human cells, known as AP-1-4; each is localized to an overlapping but different portion of the endosomal system. The AP-1 complex is found in vesicles localized to the trans-Golgi network (TGN) and recycling endosomes. The AP-2 complex is involved in internalization of clathrin-coated vesicles from the cell surface, and AP-3 is associated with trafficking between lysosomes,
endosomes, and the TGN. The AP-4 complex is the least studied, but is associated with basolateral sorting in polarized cells.

Each complex is made of four subunits: a large, specific subunit ($\gamma, \alpha, \delta, \varepsilon$ for AP1-4, respectively), a large, homologous subunit ($\beta_{1-4}$), a medium subunit ($\mu_{1-4}$), and a small subunit ($\sigma_{1-4}$). During assembly of these heterotetrameric complexes within the cell, two hemi-complexes are first assembled. The small subunit interacts with the large-specific subunit, and the $\mu$ subunit binds the $\beta$ subunit. The two large subunits then bind, connecting the two hemi-complexes into a large heterotetrameric complex ($##$). As previously mentioned in the context of Nef interactions, the $\gamma-\sigma_1$, $\alpha-\sigma_2$, and $\delta-\sigma_3$ hemi-complexes bind to motifs that contain a di-leucine motif, in Nef the $E_{160}xxxLL$ residues. Additionally, the $\mu$ subunits have been implicated in binding to tyrosine-based sorting motifs conforming to the $Yxx\phi$ sequence, where $\phi$ represents a bulky, hydrophobic residue.

**Objective of the Dissertation**

There is no doubt that the downregulation of cell-surface MHC-I by HIV-1 Nef is beneficial to the virus. Ex vivo studies have shown the ability of Nef-mediated downregulation of MHC-I to aid in the evasion of death by CTL (9). Additionally, monkeys infected with the SIV-Nef containing mutations in the regions associated with MHC-I downregulation evolved compensatory mutations to maintain MHC-I downregulation function (32).

The importance of MHC-I downregulation was further confirmed in the study presented in Chapter II. After transmission of the virus to a new host, the virus faces a novel repertoire of immune responses, creating diversifying pressures upon protein
epitopes. A way to avoid recognition of those epitopes is to mutate residues within or just outside them; however, these mutations may come at a cost to the functionality of the protein. While Nef is a main target of the early cell-mediated immune response, and displays many changes in regions associated with epitopes, the function of MHC-I downregulation is maintained across sexual transmission.

The mechanisms of action of motifs in Nef during the modulation of MHC-I are uncertain, and a major aim of this dissertation has been to dissect the mechanism by which Nef interacts with components of the endosomal sorting system and with the MHC-I cytoplasmic tail. The acidic cluster motif of Nef has been proposed as a binding partner to PACS-1, but here I show instead that this motif may be involved in binding AP-1 directly via the μ1 subunit, obviating the need for PACS-1. Additionally, I provide evidence that the N-terminal α-helix is involved in binding to the cytoplasmic tail of MHC-I, but not to the AP-1 complex. In Chapter V, I will offer thoughts on further experiments in this field that will test an elaborate model derived from these data.
References


Chapter II:
Maintenance of Nef-mediated modulation of MHC-I and CD4 after sexual transmission of HIV-1
Abstract

Viruses encounter changing selective pressures during transmission between hosts, including host-specific immune responses and potentially varying functional demands on viral proteins. The HIV-1 Nef protein performs several functions likely important for successful infection, including immune escape via down-regulation of class I major histocompatibility complex (MHC-I) and direct enhancement of viral infectivity and replication. Nef is also a major target of the host cytotoxic T lymphocyte (CTL) response. To examine the impact of changing selective pressures on Nef functions following sexual transmission, we analyzed genetic and functional changes in nef clones from six transmission events. Phylogenetic analyses indicated that the diversity of nef was similar in both sources and acutely infected recipients, the patterns of selection across transmission were variable, and regions of Nef associated with distinct functions evolved similarly in sources and recipients. These results weighed against the selection of specific Nef-functions by transmission or during acute infection. Furthermore, there was no trend toward optimization of either the CD4 or MHC-I down-regulation functions of Nef after the transmission events. Although rare clones from sources were defective in these phenotypes, these clones were not detected in the recipients. Nef-specific CTL activity was present as early as three weeks after infection and appeared to be a major evolutionary force for the diversification and adaptation of nef. Despite the changing selective pressure between the source and recipient immune systems and concomitant genetic diversity, the Nef proteins maintained robust activities for the down-regulation of MHC-I and CD4. These data suggest that both functions are important for the successful establishment of infection in a new host.
Introduction

Sexual transmission is the primary route of new HIV infections worldwide (1). Although the source partner often has a chronic infection characterized by a polyclonal viral population, acute infection in the recipient partner is characterized by a mono- or oligoclonal population (44,45). This reduction in viral diversity in acutely infected recipients compared to the chronically infected sources appears to result from a transmission bottleneck. Possible factors contributing to this bottleneck include anatomical compartmentalization of virus in the genital tract of the source partner (34), a founder effect, or a release of diversifying immune pressures, resulting in reversion to a “wild-type,” more fit, virus (13). In contrast to the reduction in the diversity of env (7,10), the diversity of nef is maintained, at least during vertical transmission, indicating either the transmission of multiple strains or the rapid diversification of the quasispecies in the new host (39). This observation may indicate that host-to-host transmission and acute infection do not exert new functional constraints upon nef relative to chronic infection. Alternatively, in comparison to env, nef may be better able to maintain function despite diversification driven by selective pressures in the new host.

The functions of Nef include direct enhancement of viral replication and immune evasion. The direct effect of Nef on viral replication appears to be multifaceted, involving optimization of signaling pathways in T cells and an increase in the infectivity of virions, mediated in part by the down-regulation of CD4 [reviewed in (8)]. The interaction of Nef with the host immune system is also multifaceted. First, Nef-mediated down-modulation of class I major histocompatibility complex (MHC-I) from the surface of infected cells
mitigates the cytotoxic T lymphocyte (CTL) response. Second, Nef itself is highly immunogenic, and MHC-I-restricted immune responses detected early in infection predominantly target the Nef protein (28). Escape mutations within these epitopes allow immune evasion and drive evolution of the nef sequence (4,11,17,20,27,31).

Interestingly, these mechanisms of immune evasion may be at odds with each other; mutations that confer escape from CTL surveillance might interfere with the down-regulation of MHC-I. Such a cost of CTL-escape has been observed in vitro by propagating HIV-1 in the presence of CTL clones specific for Nef (3). Similarly, mutations associated with CTL-escape could diminish other functions of Nef that directly affect viral replication and infectivity, such as the down-regulation of CD4. As a small protein with multiple functions, Nef might be especially sensitive to the fitness costs of mutations associated with CTL-escape (2).

While Nef-mediated evasion of the CTL-response likely contributes to the establishment and maintenance of chronic infection, the direct effects of Nef on viral infectivity and replication could be critical to the initial success of transmission. These direct virologic effects of Nef could be optimized by the transmission event via at least two mechanisms. First, the absence of CTL-mediated selection pressure in the recipient (before the onset of adaptive immunity) could allow reversion of escape mutations generated in the source, yielding a more functional nef sequence. Second, the initial lack of CTL activity could allow selection of Nef proteins that are optimized for enhancement of viral infectivity and replication at the expense of down-regulation of MHC-I. Such a trade-off in Nef-activities has been observed in the case of advanced chronic infection,
when CTL responses have presumably faltered (6), but whether it occurs during acute infection is unknown.

In this study, we examined the effect of sexual transmission on genetic adaptation in \textit{nef} and on the activities of Nef proteins. We hypothesized that CTL activity would drive diversifying selection, but that most polymorphisms maintained in vivo would minimally affect Nef-function. We also hypothesized that in the acutely infected recipients, Nef would be optimized to down-regulate CD4, a phenotype that correlates with enhancement of viral replication in primary CD4-positive T lymphocytes (19,29), whereas down-regulation of MHC-I would be optimized in chronically infected source hosts whose CTL responses were more robust. Strikingly, we observed broad CTL responses not only in the sources but also in the acutely infected recipients. Under these conditions, CTL-mediated selection pressure appeared to be a major evolutionary force for molecular diversification and adaptation of \textit{nef} in all individuals. Despite such changes, the Nef proteins from both sources and recipients maintained remarkably robust functional activities for the down-regulation of both MHC-I and CD4. Neither activity was optimized by the transmission event, and in no case could gene-wide evolution be attributed to a single Nef function. These data indicate that Nef is sufficiently adaptable to maintain two independent functions despite the changing immune pressure associated with sexual transmission.
Experimental Procedures:

Study Participants, Specimen Collection and Processing

Eleven subjects were identified through the NIH funded Acute Infection and Early Disease Research Program (AIEDRP), representing six transmission events (Pair 6 included a different recipient matched to the same donor as Pair 5). Three of the six pairs were identified by epidemiological linkage. The other three pairs were identified through screening of the AIEDRP cohort by phylogenetic analysis of baseline population-based pol sequences. Pairs that were linked through phylogenetic analysis demonstrated pol sequence homology such that transmission between the individuals was highly likely (<1% genetic divergence), as described previously (15) and below. Additionally, population based nef sequencing was performed from HIV RNA extracted from blood plasma from all suspected source and recipient partners to confirm transmissions by phylogenetic analysis (16). Study participants were separated into Acute Early HIV classes to reflect their stage of HIV infection [42].

Samples were obtained from sources from 26 days before to 45 days after the presumed date of transmission, with the exception of the source in pair 3, from whom blood was only available 627 days after transmission. Recipients were sampled 21-45 (mean 37) days after transmission. The time of infection was estimated using established AIEDRP algorithms (27).

Blood from documented source partners and the recipients was collected and centrifuged to separate peripheral blood mononuclear cells (PBMCs) and plasma. Plasma samples were stored at -80°C until analysis.
HIV-1 Plasma RNA Extraction and Sequencing

HIV RNA was extracted from blood plasma using the QiaAMP viral RNA Mini Kit (Qiagen, Valencia, CA). RNA was amplified via RT-PCR using the Finnzyme system (Finnzyme, Espoo, Finland) with primers situated outside of nef in the viral genome (Table S2, OutNef5’ and OutNef3’) followed by nested PCR with internal primers (Supplementary Table 2, nef_IN5’ and nef_IN3’) using Hi-Fidelity Platinum Taq (Invitrogen, Carlsbad, CA). To avoid contamination, source and recipient RNA was extracted and amplified on separate days and in separate locations. After purification with the Qiagen PCR cleanup Kit (Qiagen), products were cloned into the pcDNA 3.1 TOPO-V5/His vector (Invitrogen) following the manufacturer’s instructions. Individual clones were sequenced using the T7 Forward and BGH Reverse primers provided with the TOPO-V5/His kit (Invitrogen) and an ABI 3100 Genetic Analyzer. A total of 138 clones were sequenced; 8 to 28 clones for each sample of plasma RNA. Abbreviations for sequences obtained in this study use the following nomenclature: Sx or Rx, where S is for the source partner, R is for the recipient partner, and x is the Pair number as delineated in Table 1. For the phenotypic analyses, the sequences are referred to as Sx-y or Rx-y, where y refers to the clone number. Sequences used in this study are available for download in the NEXUS format from http://www.hyphy.org/pubs/NEF/sequences.zip

Sequence Analysis

Translated sequences were aligned against HXB2 nef (Pubmed ID: K03455), mapped back to corresponding codons, and manually adjusted using BioEdit. Aligned sequences were subsequently subjected to an array of maximum likelihood-based comparative analyses (described below) in an attempt to quantify the evolutionary
process governing viral evolution during transmission and adaptation to the immune response. Unless otherwise stated, all analyses were performed using the HyPhy (www.hyphy.org) (38) software package. HyPhy scripts implementing the analyses are either a part of the package distribution, or can be obtained from the authors upon request.

Clonal sequences from each transmission pair were screened for evidence of recombination. Phylogenetic trees were built using a heuristic maximum likelihood search procedure under the REV (43) substitution model with site-to-site rate variation corrected for with the beta-Gamma distribution (25), performing randomized sequential addition with nearest neighbor interchange branch swapping after every 10 sequences were added.

To confirm that transmission events were genetically linked, six phylogenetic trees were reconstructed: one separately for each transmission group, and a master tree for all transmission pairs. To assess diversity within and between transmission pairs, we estimated the mean pairwise sequence divergence within the source and the recipient of each transmission pair based on the maximum likelihood phylogeny and branch lengths derived by fitting the MG94xREV model of codon evolution (23) with the appropriate distribution of site-to-site substitution rates. Additionally, we computed approximate 95% confidence intervals on the mean divergence using profile likelihood.

**Analysis of Selection**

For each transmission pair, selection within individuals was quantified using a random effects likelihood (REL) model, which assumes that all branches within the individual’s nef sequence tree share the same dN/dS (36). For the transmission branch(es)
between each individual in a pair, selection was quantified using a newly developed genetic algorithm to account for possible heterogeneity of dN/dS within the source and recipient (26). This algorithm uses model-averaged evidence for adaptive change along the transmission branch, which avoids bias due to model mis-specification.

**Selection on discordant CTL epitopes**

The region-wide test for differential strength of selection (37) was extended to investigate whether adaptive evolution, measured both by the strength of selection (dN/dS) and the proportion of codons undergoing adaptive change (p, with dN>dS) was different within CTL epitopes present only in the source or the recipient (but not in both) compared with the rest of the sequence. First, we fitted a codon model which allowed site-to-site variation in both synonymous and non-synonymous substitution rates (35). Second, we compared whether the estimates of p and dN/dS were significantly different between the regions using the likelihood ratio test with appropriately constrained models.

**Conservation of Functional Regions**

Cumulative inter-patient amino acid divergence was estimated using only the internal branches in the phylogenetic tree that encompassed between-patient evolution, after partitioning the nef sequence into several non-overlapping regions of interest and considering the rest of the sequence as background. To measure divergence as expected amino-acid substitutions per site per unit time, we fitted the Jones et al (21) model of amino-acid evolution to each sequence region individually using pooled source and recipient sequences. This model was chosen among 12 popular empirical evolutionary models using small sample Akaike’s Information Criterion scores. Approximate 95%
confidence intervals for cumulative inter-patient divergence were determined using profile likelihood for each region of interest and the background. Lastly, we conducted a likelihood ratio test to determine whether inter-patient divergence was significantly different between the regions.

**HLA Genotyping and Mapping of Nef-specific CTL**

HLA genotyping was performed as described previously using PCR-based sequencing (30). The CTL responses were detected as described previously (28). In brief, the presence of specific CTL was detected using expanded PBMCs and an IFN-γ ELISpot assay. CD8+ cells were screened for reactivity as measured by secretion of IFN-γ using a library of 15mer peptides representing the entire Clade B consensus sequence of Nef (NIH AIDS Research and Reference Reagent Program). Spot-forming cells (SFC) were counted using an automated ELISpot reader (AID). Counts were all normalized to SFC/10⁶ cells. A response was considered significant if it was both greater than 2 times the average SFC/10⁶ cells of the negative control wells and greater than 100 SFC/10⁶ cells over the background of the negative control wells.

**Plasmid Construction for Functional Analysis**

Selected clones used for functional analyses were amplified by PCR as follows. For Pairs 1, 3, 4 and 5, the sense primer used was EcoNefpci5’; and the antisense primers were pci_S1R1_Sal3’ for Pairs 1 and 3, CF_194_SalNef3’ for Pair 4, and S5R5_SalI3 for Pair 5’. For Pair 2, sense primer pci_S2R2_ecoRI_5’ and antisense primer pci_S2R2_SalI3’ were used. Primer sequences are provided in Supplementary Table 2. Following PCR with the Hi-Fidelity Platinum Taq system (Invitrogen), the products were inserted into the pCl-neo vector (Promega) by digestion with the EcoRI and SalI sites.
(Supplementary Table 2, in bold). All plasmids were confirmed by sequencing to exclude mutations induced by the PCR cloning process.

**Transfection and Flow Cytometry**

SupT1 cells (3x10^6) were transfected during exponential growth with 20 µg of the pCI-neo vector (empty or containing Nef clones) and 2 µg of the pCG-GFP vector (a gift from Dr. Jacek Skowronschi) as a transfection marker using the Amaza Cell Kit V, protocol O-17 (Amaza Systems, Gaithersburg, MD). Cells were incubated for 24 hours after transfection, then stained with anti-CD4-APC (Becton Dickinson), and anti-HLA-A2-PE (a generous gift from Dr. David Camerini, UCI). Cells were then fixed and analyzed on a Coulter Elite flow cytometer. The average PE and APC fluorescence intensity of the GFP-positive cells was plotted. Assays were performed in duplicate and are representative of 2-6 independent experiments for each transmission pair.

**Western Blot**

Samples for analysis by western blot were taken from the same transfected cells that were analyzed by flow cytometry. Cells were suspended in loading buffer containing SDS and boiled for 10’. After resolution on a 12% denaturing polyacrylamide gel (Bio-Rad), the proteins were transferred to a nitrocellulose membrane and blotted with the following antibodies: mouse anti-tubulin (1:6000, Sigma); sheep anti-Nef (1:1500, a gift from Dr. Celsa Spina, UCSD). Detection was performed using a goat anti-mouse antibody linked to horseradish peroxidase (Bio-Rad, Hercules, CA), and a rabbit anti-sheep antibody linked to peroxidase (DAKO, Glostrup, Denmark), followed by development with the enhanced chemiluminescence (Amersham-Pharmacia, Piscataway, NJ).
**Results**

**Characteristics of the transmission cohort.**

To examine the evolution of *nef* after sexual transmission, we identified six transmission pairs from the San Diego site of the Acute Infection and Early Disease Research Program (AIEDRP) based on epidemiologic evidence and phylogenetic analysis of the *pol* gene [(16); Table II.1]. These data suggested that two recipients were infected from the same source (see also Figure 1, pair 5/6). The average age of the subjects was 40.6 years (range 36-49), and the average CD4+ T lymphocytes/ml blood was 547.8 (range 383-930, Table II.1). A single sample of plasma RNA was analyzed from each individual; the concentrations ranged from $10^{4.4}$ to $10^{7.2}$ copies per ml (Table II.1). The estimated intervals between the transmission events and the dates of sample collection are indicated in Table 1. Notably, two recipients, R4 and R6, were sampled within 21 days of the estimated date of transmission. At the time of sampling, none of these individuals had received antiretroviral therapy.

**Phylogenetic linkage of transmission pairs by sequence analysis of *nef*.**

We analyzed nucleotide sequences of 138 *nef* clones from the six source and recipient pairs, with an average of 12 clones/individual, by cloning RT-PCR products derived from plasma RNA. Of all 138 clones, only two (both found in recipients) encoded Nef proteins predicted to be defective based on the presence of premature termination codons. The predicted amino acid sequences of the 136 intact open reading frames are shown aligned in Figure II.1, A-E.
Table II.1: Demographics of the Transmission Cohort

<table>
<thead>
<tr>
<th>Pair</th>
<th>Subject&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AEH Class</th>
<th>Age</th>
<th>CD4 Count&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BP vRNA (log)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Estimated Days between Sample/Transmission Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1</td>
<td>E1</td>
<td>49</td>
<td>476</td>
<td>6.22</td>
<td>-26</td>
</tr>
<tr>
<td></td>
<td>R1</td>
<td>A3</td>
<td>37</td>
<td>383</td>
<td>4.88</td>
<td>+45</td>
</tr>
<tr>
<td>2</td>
<td>S2</td>
<td>E2</td>
<td>41</td>
<td>599</td>
<td>5.04</td>
<td>+101</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>A3</td>
<td>38</td>
<td>562</td>
<td>4.85</td>
<td>+45</td>
</tr>
<tr>
<td>3</td>
<td>S3</td>
<td>C</td>
<td>36</td>
<td>324</td>
<td>4.43</td>
<td>+627</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>A3</td>
<td>44</td>
<td>634</td>
<td>5.12</td>
<td>+45</td>
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<td>4</td>
<td>S4</td>
<td>C</td>
<td>36</td>
<td>469</td>
<td>4.68</td>
<td>+45</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td>A1</td>
<td>35</td>
<td>466</td>
<td>6.42</td>
<td>+21</td>
</tr>
<tr>
<td>5</td>
<td>S5/6</td>
<td>A1</td>
<td>36</td>
<td>536</td>
<td>5.75</td>
<td>-73 (to R5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-49 (to R6)</td>
</tr>
<tr>
<td>6</td>
<td>R5</td>
<td>A3</td>
<td>48</td>
<td>930</td>
<td>7.18</td>
<td>+45</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>A1</td>
<td>44</td>
<td>647</td>
<td>6.70</td>
<td>+21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Subjects are homosexual men enrolled in the San Diego AIEDRP cohort.  
<sup>b</sup>Length of infection was estimated using a previously described algorithm (42).  
<sup>c</sup>CD4+ cells/µl blood.  
<sup>d</sup>vRNA = viral RNA, copies/ml blood.
Phylogenetic analysis (Figure II.2) indicated that the sequences from each individual and transmission pair formed distinct monophyletic groups, with strong bootstrap support for separating branches, with the exception of recipients R5 and R6, who grouped together and were infected by the same source (S 5/6). This tree-morphology was consistent with the putative transmission events. Recombination, which can severely bias phylogeny-based comparative methods (40), was not detected in any of the six transmission pairs, based on a robust maximum likelihood GARD test (24) (data not shown).

Importantly, in each of the six transmission events only a single branch connected the source with the recipient sequences (Figure 1). These data suggest that a mono- or oligo-clonal population of nef sequences was transmitted. In one pair (pair 4), a single source clone grouped with the recipient clones, suggesting the possibility of genome-wide purifying selection during the transmission event.

**Genetic diversity of nef in sources and recipients.**

Previous data suggested that the genetic diversity of nef is maintained across vertical transmission (39). To determine whether this finding was true of sexual transmission, the diversity of nef sequences within each host (defined as the average length of the path connecting two sequences from a given individual in the phylogenetic tree) was assessed for each transmission pair. As shown in Figure 1, the diversity of nef sequences within each individual was relatively low compared to the diversity between transmission pairs [0.16-1.80% vs. 16.22% (95% CI 14.66-17.87%), Figure II.2]. With the exception of pair 3, in which diversity was lower in the recipient, the diversities of the
Figure II.1A. Nef amino acid sequences from transmission pair 1. Nef protein sequences from each pair were aligned to the HXB2 reference sequence. Gaps are indicated by dashes. The number in subscript next to the single letter amino acid abbreviation represents the number of clones from that individual that had identical amino acid. Boxed residues indicated differences between the source (S) and recipient (R) that are conserved across all of the clones obtained from the individuals.
**Figure II.1B. Nef amino acid sequences from transmission pair 2.** Nef protein sequences from each pair were aligned to the HXB2 reference sequence. Gaps are indicated by dashes. The number in subscript next to the amino acid single letter abbreviation represents the number of clones from that individual that had the indicated amino acid. Boxed residues indicated differences between the source (S) and recipient (R) that are conserved across all of the clones obtained from the individuals.
Figure II.1C. Nef amino acid sequences from transmission pair 3. Nef protein sequences from each pair were aligned to the HXB2 reference sequence. Gaps are indicated by dashes. The number in subscript next to the amino acid single letter abbreviation represents the number of clones from that individual that had the indicated amino acid. Boxed residues indicated differences between the source (S) and recipient (R) that are conserved across all of the clones obtained from the individuals. Regions underlined are those that triggered CTL responses in functional assays.
**Figure II.1D. Nef amino acid sequences from transmission pair 4.** Nef protein sequences from each pair were aligned to the HXB2 reference sequence. Gaps are indicated by dashes. The number in subscript next to the amino acid single letter abbreviation represents the number of clones from that individual that had the indicated amino acid. Boxed residues indicated differences between the source (S) and recipient (R) that are conserved across all of the clones obtained from the individuals. Regions underlined are those that triggered CTL responses in functional assays.
Figure II.1E&F. Nef amino acid sequences from transmission pairs 5 & 6. Nef protein sequences from each pair were aligned to the HXB2 reference sequence. Gaps are indicated by dashes. The number in subscript next to the amino acid single letter abbreviation represents the number of clones from that individual that had the indicated amino acid. Boxed residues indicate differences between source (S) and recipient (R) that are conserved across all of the clones from the individuals.
nef sequences were statistically indistinguishable between the sources and the recipients of each pair.

**Diversifying and purifying selective pressures on nef.**

To examine the selective pressures in the transmission pairs and within individuals, the ratio of synonymous to non-synonymous substitution rates (dN/dS) was estimated from the entire nef gene (Figure II.2)(14). Distinct patterns of selection were detected in most pairs. Purifying selection was detected across the transmission branch of pair 1 [dN/dS=0.26, 95% confidence interval (CI) 0.1-0.32], but within either individual evolution was essentially neutral. In contrast, in pair 2, diversifying selection was found both within the source (dN/dS=2.6, 95% CI 0.9-5.6) and the recipient (dN/dS=5.1, 95% CI 2.6-9.0), while selection across the transmission branch was essentially neutral. Pair 3 exhibited trends toward purifying selection within the source and the recipient, but diversifying selection across transmission. However, this branch incorporates 18 months of sequence evolution in the source following transmission, rendering interpretation problematic. In pair 4, sequences in the recipient were under diversifying selection (dN/dS = 3, CI 1.2-6.0), while those in the source and across the transmission branch were under purifying selection (source dN/dS=0.2, 95% CI 0.1-0.5; branch dN/dS=0.5, 95% CI 0.05-1.0). Finally, in pairs 5 and 6, diversifying selection was detected across the transmission branch (dN/dS=6.2, CI 1.6-9.5), while evolution in the source (S5/6) and recipients (R5 and R6), was essentially neutral. In summary, the patterns of selection pressures on nef were pair-specific, a result that may reflect unique combinations of CTL-driven selection within and between these hosts. Purifying selection across the transmission events was not consistently found, weighing against a scenario in which
Figure II.2. Maximum likelihood phylogeny of nef sequences reveals transmission events. A maximum likelihood phylogenetic tree was constructed with all nef sequences obtained from each individual in the study. Duplicate sequences were not included. All transmission events grouped together with 100% bootstrap support. Diversity of all clones within individuals is indicated. Transmission branch length represents the genetic distance between the most recent common ancestors of the clonal populations in two individuals of a transmission pair.
the release of CTL pressure in the non-immune recipient results in convergent evolution towards a more fit nef sequence.

**Functional regions within Nef evolve at similar rates in sources and recipients.**

We initially hypothesized that in acutely infected recipients, Nef would be optimized to enhance viral infectivity and replication, while in chronically infected sources, the down-regulation of MHC-I would be optimized. To test this hypothesis, we focused the analysis of protein diversification on regions of Nef specifically associated with these functions, to determine whether any of these regions were under different rates of evolution in sources compared to recipients. The inter-patient branches of the tree were assessed between all recipients and all sources and the rate of amino-acid substitutions in specific functional regions were compared to the background rate for the rest of the protein. Three regions of Nef were assessed: 1) the N-terminal α-helix, spanning residues 16-22 (of HXB2 Nef); 2) the acidic cluster/polyproline region, spanning residues 61-80; and 3) the C-terminal flexible loop, spanning residues 143-181. The N-terminal α-helix and the acidic cluster/polyproline region are required for the down-regulation of MHC-I, whereas the C-terminal flexible loop is required for the down-regulation of CD4 and the enhancement of viral infectivity and replication in primary cultures [reviewed in (8)]. No differences in the rates of evolution in these regions between sources and recipients were detected, and sequences from both groups conformed to previously noted conservation estimates [Table II.2 and (18)]. The acidic cluster/polyproline region evolved at a rate similar to the background for the rest of the protein [excluding the other functional
### Table II.2. Relative rate of evolution of Nef sequences.

<table>
<thead>
<tr>
<th>Region</th>
<th>Sources</th>
<th>Recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Terminus α-helix</td>
<td>3.25a (1.68-5.66) p=0.002</td>
<td>2.35 (1-4.63) p=0.05</td>
</tr>
<tr>
<td>Acidic Cluster/PxxP region</td>
<td>0.66 (0.28-1.28) p=0.27</td>
<td>0.66 (0.26-1.34) p=0.86</td>
</tr>
<tr>
<td>C-Terminal Loop</td>
<td>1.66 (1.15-2.31) p=0.02</td>
<td>1.64 (1.1-2.4) p=0.025</td>
</tr>
<tr>
<td>Rest of Nef protein</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*aNumbers represent the relative rate of evolution of specific regions of Nef (using rest of sequence as ‘1’) along interpatient branches in the trees, fitted using the JTT model of protein evolution, along with 95% confidence intervals in parentheses. P-values indicate whether those rates are different among the functional region versus the rest of nef, not including the other functional regions.*

### Table II.3. HLA-Genotypes and CTL responses of Transmission Pairs

<table>
<thead>
<tr>
<th>HLA-A</th>
<th>HLA-B</th>
<th>HLA-C</th>
<th>Regions in Nef* Triggering CTL Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>01_03</td>
<td>40_08</td>
<td>03_07 137-151, 149-163</td>
</tr>
<tr>
<td>R1</td>
<td>01_02</td>
<td>13_08</td>
<td>06_07 95-109</td>
</tr>
<tr>
<td>S2</td>
<td>30_11</td>
<td>70_14</td>
<td>07_08 69-95</td>
</tr>
<tr>
<td>R2</td>
<td>02_11</td>
<td>15_44</td>
<td>30_05 No response detected</td>
</tr>
<tr>
<td>S3</td>
<td>02_24</td>
<td>39_48</td>
<td>08_12 77-95, 109-123, 177-203</td>
</tr>
<tr>
<td>R3</td>
<td>30_33</td>
<td>44_70</td>
<td>07_07 69-87, 89-103, 129-143</td>
</tr>
<tr>
<td>S4</td>
<td>01_02</td>
<td>53_57</td>
<td>04_06 131-146</td>
</tr>
<tr>
<td>R4</td>
<td>01_11</td>
<td>08_38</td>
<td>07_12 69-83, 81-103, 105-119, 177-199</td>
</tr>
<tr>
<td>S5/6</td>
<td>32_68</td>
<td>40_44</td>
<td>03_07 5-23, 113-127</td>
</tr>
<tr>
<td>R5</td>
<td>24_24</td>
<td>15_44</td>
<td>03_05 NA*</td>
</tr>
<tr>
<td>R6</td>
<td>11_29</td>
<td>44_44</td>
<td>16_16 NA*</td>
</tr>
</tbody>
</table>

*aNumbering refers to the HIV-1 clade B Nef consensus sequence. bNA = responses not available due to lack of viable samples.*
regions analyzed (Table II.2). In contrast, the N-terminal α-helix and the C-terminal flexible loop evolved at a significantly higher rate than the rest of the protein (Table II.2), suggesting that these regions can tolerate a high degree of heterogeneity. Importantly, key residues in these regions, such as the central prolines in the polyproline helix and the two leucine residues in the C-terminal loop, were universally conserved. Overall, the rates of evolution in regions of Nef related to specific functions did not appear to change measurably with transmission (Table II.2), suggesting that differential selection of a specific function across transmission or during acute infection may not occur.

**CTL responses to Nef peptides were divergent between individuals and may drive diversification after transmission.**

Many of the amino acid differences between the source and recipient clones, and between these clones and the HXB2 reference sequence (Figure II.1), were previously described as escape adaptations to CTL-activity (3,6,39). To test the hypothesis that these polymorphisms may be attributed to the selective pressure of CTL, both the HLA genotypes of the individuals and the breadth of their CTL activity were determined (Table II.3 and Figure II.1). For the detection of CTL activity, peripheral blood mononuclear cells obtained at the same time as the plasma RNA samples were expanded and responses were measured using an array of overlapping peptides that covered the entire Nef protein in an ELISpot assay for IFN-γ (Table II.3).

CTL responses to clade B consensus Nef peptides were detected in 8 of 11 individuals. Two of the individuals were unable to be tested due to a lack of viable cells, and in individual R2, no responses were detected despite viable cell cultures. Notably,
two individuals in the earliest stage of infection exhibited broad CTL responses (R4 and S5/6).

To assess the possibility that CTL activity was a driving force in the diversification of nef sequences across transmission and during acute infection, we took advantage of the observation that pair 3 exhibited fairly divergent and broad CTL responses (Table II.3, Figure II.1C). This allowed the comparison of selection within regions that were only targeted by the CTL of one individual (discordant epitopes) with selection elsewhere in the Nef protein. Accordingly, the 15% of the codons that were in discordant epitopes were under strong diversifying selection (dN/dS = 8.4). Of the codons outside discordant epitopes, 54.8% were under weak diversifying selection (dN/dS=1.5), while the remaining codons were not under detectable selection. This difference was significant (p<0.01), indicating that changes in CTL recognition between hosts can be a major driving force for the diversification of nef sequences during transmission.

**Functional effects of Nef variations in transmission pairs.**

To examine the impact of observed amino acid polymorphisms on function, clones from all individuals were assayed for functions related to immune evasion (down-regulation of MHC-I) as well as down-regulation of CD4, a surrogate marker for enhancement of replication-rate (29). The clones selected for analysis met at least one of the following criteria: 1) the clone encoded the amino acid sequence closest to the consensus sequence for that individual; 2) the clone contained one or more mutations found in at least two other clones; or 3) the clone contained
Figure II.3. Sequences of clones used in phenotypic studies. S= sources, R= recipients. The number after S or R indicates the pair, the number after that designates the clone, shading separates transmission pairs. Sequences are aligned to HXB2; dots for identity and dashes for gaps. Bold boxes show differences within a pair. 1The consensus sequence for that individual; 2Changes found in a minority population; 3the clone encoded mutations in an amino acid sequence near a known functional region of Nef.
mutations in or near a known functional region of Nef. An alignment of the tested clones is shown in Figure II.3.

Overall, 21 Nef-encoding clones from the six transmission pairs were assessed for the down-regulation of MHC-I and of CD4 using transiently transfected SupT1 T cells and a flow-cytometric assay (Figure II.4). The expression of the various Nef proteins in the transfected cells was tested by western blot using a polyclonal antiserum. Of the 21 clones, four (S1-3, S1-4, S3-2, and S3-4) exhibited functional defects. In three of these cases, the defects could be attributed to poor expression (Figure II.4A and C). None of these four functionally impaired source clones represented the majority variant in that host.

In general, both the down-regulation of MHC-I and of CD4 were well maintained in both sources and recipients. No overall enhancement or diminution of either Nef-function was detected between source and recipient sequences. As noted above, certain minority clones encoded defective or partly defective proteins. Clone S1-3 was selectively impaired in the down-regulation of CD4 and contained a unique mutation, V166G (Figure II.3), located near the N-terminal end of the C-terminal flexible loop. Although apparently poorly expressed, this clone maintained robust down-regulation of MHC-I (Figure II.4A). Clone S1-4 was completely nonfunctional for CD4 or MHC-I down-regulation, and the expression of Nef was not detected by western blot, possibly due to V159A and/or W196R mutations. The W196R mutation was not found in other clones of the cohort, while the V159A mutation was found in single clones from R4 and S5/6 not analyzed in Figure 3 (Figure II.1, D and E). Minority clones derived from S3 also exhibited defects in Nef functions (S3-2 and S3-4, Figure II.4C). The S3-2 clone was
minimally active in the down-regulation of MHC-I, and it had an intermediate ability to
down-regulate CD4. The S3-4 clone was virtually inactive for either function. These
defects were not clearly attributable to specific, unique polymorphisms. However, both
S3-2 and S3-4 encoded a substitution of serine 8 with leucine, and S3-4 encoded a
substitution of serine 116 with proline; these mutations were not found in other clones of
the cohort. The S3-3 and R3-1 clones were phenotypically wild-type, despite relatively
low levels of protein detected by western blot, suggesting antigenic differences that
hindered the detection of these Nef proteins.

The remaining 17 of 21 clones analyzed were fully functional in both the down-
regulation of MHC-I and of CD4. These data suggest that both of these Nef functions are
important during HIV transmission and acute infection. The data also indicate that Nef is
able to tolerate extensive and diverse polymorphisms, some of which are likely responses
to the selective pressure of CTL, without compromising its immune evasion and virologic
functions.
Figure II.4. Phenotypic analysis of Nef clones from the transmission pairs: down-regulation of MHC-I and CD4. Clones from each pair, described in Figure 3, were used to transfect SupT1 T Cells along with a plasmid encoding GFP as a transfection marker, and the cells analyzed by three-color flow cytometry. The mean fluorescence intensities (MFI) of GFP-positive cells are graphed for CD4 (light gray bars) and HLA-A2 (darker gray bars). Each experiment was performed in duplicate; error bars represent one standard deviation. Beneath each graph is a western blot of the same cells used in the flow cytometry experiments probed for Nef using a polyclonal antiserum. Duplicates from each experiment were analyzed; a representative blot is shown.
Discussion

In this study, we explored the genetic and phenotypic changes in the HIV-1 nef gene in epidemiologically and phylogenetically linked transmission pairs. The transmission cohort represented a range of stages of HIV-1 infection. In principle, this allowed a test of the hypotheses that specific Nef-sequences and functions were associated with transmission or were optimized during acute infection. Sequence and phylogenetic analyses indicated that although the diversity of the nef sequences in acutely infected recipients was usually similar to that in their matched sources, tree morphology suggested that a mono- or oligo-clonal population of nef sequences was transmitted. The patterns of gene-wide selection across transmission were variable, and regions of Nef associated with distinct functions evolved similarly in sources and recipients. These results weighed against the selection of specific Nef-functions by transmission or during acute infection. This conclusion was supported by direct measurement: the relative activities of down-regulation of CD4 and MHC-I, properties with distinct genetic requirements, were virtually unchanged by the transmission of HIV-1 between hosts. Furthermore, in the majority of clones in both sources and recipients, these two functions were remarkably well preserved despite extensive sequence diversity.

In this cohort, the nef coding region varied by as much as 18% between transmission pairs but exhibited substantially lower diversity within individuals and within pairs. The diversity within the samples obtained from the individuals of each pair did not differ significantly, with one exception (pair 3) in which diversity decreased after transmission. The lack of a consistent reduction in the diversity between the individuals of each pair might suggest the absence of a genetic bottleneck during sexual transmission
with respect to nef. As noted previously in the case of vertical transmission (39), this observation may reflect either the transmission of multiple viral strains or the rapid diversification of a mono- or oligoclonal population in the new host. In support of the latter, the diversity of nef sequences correlated to some extent with the duration of infection in the recipient (Figure II.2 and Table II.1). For example, the recipients in pairs 1, 2 and 3 were in the later stages of acute infection (A3) and their Nef sequences were characterized by relatively high diversities compared to the recipients of pairs 4 and 5/6, who are in the earliest stages (A1). Pairs 1, 2, and 3 also have longer transmission branches than pairs 4 and 5/6, consistent with the hypothesis that diversity increases with time during acute infection.

The different patterns of selection (purifying versus diversifying) observed among the transmission pairs may reflect various pairings of concordant and discordant immune responses. The variability in selective pressures after transmission observed here in the case of nef is reminiscent of a characterization of evolution within env as driven by neutralizing antibodies (14). Under these variable conditions, the CTL response could drive either purifying or diversifying selection. For example, transmission could drive purifying selection if CTL escape mutations generated in the source confer a selective advantage in the recipient due to recognition of the same epitopes. A possible example of this is found in pair 1; the individuals of this pair share three HLA alleles, and purifying selection was inferred across the transmission branch. A more common scenario may be one in which diversifying, positive selection results from novel immune responses in the recipient relative to those in the source. While pairs 5 and 6 may represent such cases (diversifying selection occurred across transmission) the CTL response was unable to be
evaluated in the recipients due to the unavailability of viable cells. Notably, positive selection was detected in sequences obtained from individuals S3 and R4, both of whom had broad CTL responses, a result consistent with the hypothesis that CTL pressure drives diversifying selection.

In addition to the selective pressures induced by host-to-host variation in CTL activity, the process of transmission might select for a specific Nef-function, for example, the enhancement of viral infectivity or replication. Genetic evidence of such selection was sought by examining the relative rates of amino acid evolution in regions of Nef associated with specific functions: either the down-regulation of MHC-I (the N-terminal α-helix and the acidic cluster/polyproline region) or the down-regulation of CD4/enhancement of infectivity and replication (the C-terminal flexible loop). The estimated rates of evolution of these regions were essentially the same in sources and recipients, providing genetic evidence against the association of a specific Nef-function with the efficiency of transmission or with viral replication in the acutely infected host. Consistent with previous data, the increased rate of evolution within the N-terminal α-helical region and the C-terminal flexible loop suggested that these areas are relatively more tolerant of polymorphisms than the acidic cluster/polyproline region (22,41).

The diversity of Nef sequences and specific polymorphisms within this cohort were extensive, yet the majority of the Nef proteins tested were fully functional and previously defined key residues were conserved. For example, an N-terminal duplication (Q33-A38, pair 2; analogous to previously noted duplications found in 36% of available nef sequences) did not affect function (18). A key position in this region associated with the down-regulation of MHC-I (M20) was conserved or contained a conservative change.
(I20). The acidic cluster region associated with the down-regulation of MHC-I (E62-65 in HXB2; positions 73-78 in Figure II.3) exhibited insertions and changes that did not affect function; this observation is consistent with the reported insertion of a glycine or glutamic acid in the acidic cluster motif in ~5% of nef sequences (18). An additional acidic residue in this motif did not confer improved activity in MHC-I down-regulation (compare S2-1 and S2-2 to S2-3). Notably, all the prolines of the SH3-binding region were conserved throughout this cohort. The variability of positions within the C-terminal flexible loop was extensive. The di-acidic sequence associated with binding to β-COP (5,33) had a high proportion of lysine substitutions at the second glutamic acid [position 168 in Figure 2; also seen in (22,41)]; the majority of these clones were fully functional (all tested clones of pairs 2, 4, and 5/6, as well as clone S3-3). Non-conservative substitutions within the acidic di-leucine motif (E160xxxLL165 in HXB2; positions 173-178 in Figure 2) demonstrated unexpected flexibility at the +2 and +3 positions relative to the acidic residue. These polymorphisms included the replacement of polar, uncharged residues with hydrophobic and basic residues, substitutions that were surprisingly functional in view of the sequence preferences for the binding of such motifs to the adaptor protein (AP) complexes involved in endosomal trafficking (9). Nevertheless, the ExxxLL AP-binding motif within the C-terminal flexible loop was universally conserved in this cohort. Finally, the F191 residue recently associated with binding of Nef to Pak2 (position 204 in Figure II.3) was also universally conserved (32).

As noted above, we initially hypothesized that the ability of Nef to down-regulate CD4 would be optimized in acutely infected patients at the expense of its ability to down-regulate MHC-I. This hypothesis was based on the assumption that subjects identified
very early during acute infection would essentially be non-immune. It also followed from the reported relationship between these two Nef-activities in late-stage, chronically infected patients whose CTL responses had presumably waned: a relative optimization of the down-regulation of CD4 at the expense of down-regulation of MHC-I (22). This hypothesis was not supported; both functions were robust in the majority of clones, and no change was detected in either activity across the transmission event. Why would the modulation of MHC-I be preserved in these acutely infected individuals? We suspect that at the time of sampling, both sources and recipients had developed CTL responses, which would provide a selective advantage to Nef proteins able to down-regulate MHC-I. Indeed, broad CTL activity was detected as early as 21 days after transmission (individual R4), suggesting that the temporal window during which Nef can evolve in the non-immune, acutely infected host is very short.

Because we have not measured all the activities of Nef, it remains possible that a property of Nef so far untested is optimized during transmission or acute infection. Such properties include the ability of Nef to enhance viral infectivity or replication by a mechanism independent of the down-regulation of CD4, or the ability of Nef to facilitate T cell activation. In this regard, we are aware of no residue that has been described as important for either of these Nef-phenotypes that is not also important for either the down-regulation of CD4 or MHC-I. Taken together, the two genetically distinct Nef-functions measured here may serve as an effective general screen for defects in Nef-activity.

In conclusion, two distinct Nef-functions, the down-regulation of CD4 and of MHC-I, were well conserved both before and after sexual transmission of HIV-1. Genetic
and phenotypic data suggested that Nef tolerates multiple mutations, probably driven by CTL-pressure, without a fitness-cost. This conclusion is consistent with a recent report indicating that CTL escape variants of internal viral proteins are transmitted sexually in proportion to their frequency in the source; no general diminished capacity for transmission was detected (12). The data also suggest that neither sexual transmission nor the subsequent acute infection subjects Nef to functional constraints distinct from those present during chronic infection. We speculate that the down-regulation of CD4 and MHC-I by Nef are each crucial to the successful establishment and maintenance of a new infection. Alternatively, these two activities may together reflect an as yet unrecognized overarching function of Nef that is preserved throughout various stages of disease.

Acknowledgements

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Lewis, DD Richman, SK Pillai, OO Yang, SJ Little, DM Smith, and JC Guatelli. *Journal of Virology*, 2007. doi: 10.1128/JVI.01793-06. The dissertation author was the primary investigator and author of these papers.
References


Chapter III:

The role of ADP-Ribosylation Factor 6 in Nef-mediated Downregulation of MHC-I
Introduction

MHC-I downregulation by Nef was first thought to follow a similar mechanism to that of CD4 downregulation by Nef; an increase in the rate of MHC-I endocytosis from the cell surface appeared to account for relocalization to the juxtanuclear region (3,10). The logical place to begin studying which cellular proteins were involved in MHC-I downregulation was the endocytic compartment that MHC-I would normally utilize. Extensive work by Julie Donaldson and colleagues on nonclathrin endocytosis showed that MHC-I was internalized into a compartment modulated by ADP-Ribosylation Factor 6 (ARF6), and thus my initial efforts at understanding MHC-I downregulation were focused upon this protein.

ARF6 is a small GTPase involved in membrane trafficking. While ARF6 circulates between the plasma membrane and an early, nonclathrin recycling compartment, other ARF family members (ARF1, ARF3) are located in or near the Golgi apparatus (7). ARF6 is unique from other ARFs in that it is constitutively associated with membranes, whereas ARF1 and ARF3 cycle on and off membranes dependent upon their GTP cycle. However, like the others, the GTP cycle of ARF6 determines its localization; when GTP-bound, ARF6 is found at the plasma membrane, and when GDP-bound it is localized to an internal compartment (7,8). The coat components of ARF6 vesicles are unknown; however, it is known that clathrin, the AP complexes, and the COP proteins are not found in purified ARF6 vesicles (7).

ARF6 is involved in actin cytoskeletal rearrangements, phagocytosis, and macropinocytosis. Cell-surface proteins found in the internalized compartment created by ARF6 endocytosis include the IL-2 receptor (Tac) and the MHC-I complex (8). As HIV-1
Nef had been shown to increase the rate of endocytosis of MHC-I (10), we sought to address the question of whether ARF6 was involved in Nef-mediated downregulation of MHC-I. To explore this hypothesis, we utilized mutants of ARF6 that were “locked” in the either the GDP-bound state (ARF6T27N) or the GTP-bound state (ARF6Q67L). Using these mutants, we hoped to examine the role of ARF6 in Nef-mediated downregulation of MHC-I.
Experimental Procedures

Cell Culture.

HeLa cells were maintained in DMEM with 10% fetal bovine serum, glutamate, and streptomycin/penicillin. Jurkat T cells were maintained in RPMI plus 10% fetal bovine serum, glutamate, and streptomycin/penicillin.

Plasmids and Constructs.

The ARF6-HA, ARF6Q67L-HA, and ARF6T27N-HA constructs, all in the pXS vector, were obtained from Julie Donaldson. Constructs encoding NefGFP, used in the immunofluorescence microscopy, have been previously described (2). The pC1-neo vector containing NL4-3 Nef, and the pCG-GFP vector, used in the flow cytometry experiments, have also been previously described (1).

Immunofluorescence Microscopy.

6x10^4 HeLa cells were plated on 12mm glass cover-slips in a 24-well dish 16 hours before transfection. Transfections were performed with the Fugene reagent and 1µg total of DNA, as per manufacturer’s instructions. After 24 hours, cells were washed, fixed, permeabilized and stained with antibodies against the HA-epitope (Zymed) to detect the ARF6 protein and against the HLA-A,B,C complexes (Clone W6.32, Upstate). Coverslips were inverted on mounting medium, and processed using a Zeiss Confocal Microscope at the National Center for Microscopy and Imaging Resource at UC San Diego.
**Flow Cytometry.**

3x10^6 Jurkat T cells were transfected per sample using the Amaxa Nucleofector Kit V. Each sample was transfected with a total of 10 µg of plasmid DNA. After 24 hours, cells were washed and stained with a PE-conjugated antibody to HLA-A,B,C (Pharmigen). Cells were fixed and analyzed by the VA Flow Cytometry Core.
Results

The first objective of these studies was to ascertain whether Nef and ARF6 might encounter each other in an intracellular compartment. I performed immunofluorescence microscopy on HeLa cells transfected with plasmids expressing NefGFP or ARF6 tagged with the HA epitope. Indeed, vesicles containing not only ARF6-HA and Nef-GFP, but also MHC-I were present in the juxtanuclear region (Figure III.1, white puncta in Merge).

Next we sought to understand whether perturbations of the GTP cycle of ARF6 would affect the ability of Nef to modulate MHC-I at the cell surface of Jurkat T cells. Previous characterization of ARF6 led to the discovery of mutants that could mimic the different forms of ARF6 during its GDP/GTP cycle. The Q67L mutant mimics the GTP-bound form of ARF6, while the T27N mutant mimics the GDP-bound form. Cells were transfected with plasmids expressing Nef and either ARF6-HA, ARF6Q67L-HA, or ARF6T27N-HA, as well as GFP as a cotransfection marker. Theoretically, if ARF6 is necessary for MHC-I internalization, and if Nef used this pathway to increase endocytosis of MHC-I, then perturbing the cycle would have an effect upon Nef-mediated downregulation of MHC-I. Interestingly, coexpressing a GTP-bound form of ARF6 appeared to inhibit the internalization of MHC-I in the presence of Nef (Figure III.2 A, Nef+ARF6Q67L vs. Nef+ARF6, p=0.05, Student’s T-test).

However, these results were difficult to interpret, as effects of GTP-cycle mutations might affect MHC-I cycling in general, and not the Nef-effect specifically. To test this possibility, we examined cell-surface expression of MHC-I in the presence of the ARF6 mutants without Nef in Jurkat T cells (Figure III.2 B). The GTP-locked mutant
appeared to increase cell-surface expression of MHC-I, but was not significantly different from the Mock-transfected sample. From these data, it appears that the expression of the ARF6 mutants without Nef does not disturb the expression of MHC-I.
Figure III.1: Arf6 colocalizes with NefGFP. GFP, Nef-GFP, HA, Arf6-HA, MHC, endogenous MHC-I.
Figure III.2: Perturbations of the ARF6 GTP cycle have a general effect on MHC-I trafficking. The PE mean fluorescence intensity of GFP+ cells is plotted as a reflection of MHC-I-A,B,C expression. “Mock” refers to empty-vector control. *indicates p<0.05 by Student’s T-test. A, Jurkat T cells coexpressing Nef and Arf6 mutants. B, Arf6 mutants alone.
Discussion

During these studies, two papers were published on the role of ARF6 in Nef-mediated downregulation of MHC-I. The first, Blagoveschenskaya et al., Cell 2002, purported that ARF6 was activated by Nef, and this activation, which increased the rate of turnover of the ARF6-GTP/GDP cycle, increased the amount of MHC-I internalized. The second, published two years later, stated that Nef-mediated downregulation of MHC-I was independent of the ARF6 cycle (6). Our data regarding the perturbations upon MHC-I cell-surface expression in the presence of ARF6 mutants appear more consistent with the first study. Thus, we conclude that MHC-I likely traffics through an ARF6-mediated compartment, and that this pathway may be utilized by HIV-1 Nef to reduce cell-surface expression.

During the time of the studies on ARF6, I also examined protein-protein interactions between Nef and the clathrin adaptor protein complexes (AP1-4), with a focus upon AP-1. Curiously, several studies have argued that the majority of the Nef-effect upon MHC-I cell surface expression occurs before the MHC-I traffics to the cell surface; that is, it is redirected from the exocytic pathway to a juxtanuclear compartment (4,5). AP-1 is involved in endocytic trafficking from the trans-Golgi network to endosomal vesicles, and a large concentration of AP-1 is localized on juxtanuclear vesicles, similar to the distribution of Nef and MHC-I (Figure III.I). A crucial link between Nef, AP-1 and MHC-I trafficking came in the form of a study published by the Collins lab (9), providing evidence via RNA interference that AP-1 was involved in downregulation of MHC-I. Thus, as described in the next chapter, I refocused my efforts to study this interaction.
References


Chapter IV:

The cooperative binding of the MHC-I cytoplasmic domain and HIV-1 Nef to the endosomal adaptor AP-1
Abstract

The mechanism by which HIV-1 Nef reduces the cell-surface expression of MHC-I is incompletely defined. Previous data point to a direct interaction between Nef, the cytoplasmic domain of MHC-I, and the AP-1 complex. However, the actual points of interaction between these three components are unknown. Furthermore, if Nef requires AP-1 to modulate MHC-I, why is the canonical AP-1 binding motif in Nef, the ExxxLL_{160-165} sequence, not required to modulate MHC-I? We examined the possibility that Nef contains an alternative AP-1 binding site that is revealed when complexed with the MHC-I-A2 cytoplasmic domain (CD). A synergistic interaction between Nef and the MHC-I-A2 CD was identified that increased the affinity for AP-1 and was independent of the canonical AP-binding motif in Nef. However, this interaction did required the Y_{320} residue of the MHC-I-A2 CD, and the EEEE_{62-65} and P_{78} residues of Nef. In an in vitro system excluding other cellular proteins, the μ1 subunit of AP-1 was sufficient for this interaction. We conclude that the Nef protein acts in concert with the MHC-I CD to create a novel interaction with the μ1 subunit of AP-1, and that these three components are sufficient to explain the retention of MHC-I by Nef within the endo-lysosomal system.
Introduction

HIV-1 evades the host immune system by reducing the amount of major histocompatibility class I (MHC-I) on the cell surface, thereby inhibiting host cell lysis by cytotoxic T lymphocytes (CTL)(10,24). The viral protein associated with this function is the HIV accessory protein Nef, which is also known to modulate several other cell surface proteins, including the HIV receptor CD4, the dendritic cell lectin receptor DC-SIGN, MHC-II, and the transferrin receptor, among others (8,17). The exact mechanism by which Nef reduces cell-surface MHC-I remains controversial. Some data support the idea that the decrease in cell-surface expression of MHC-I is a result of increased endocytosis, while other data suggest that the majority of the effect can be attributed to an inhibition of transport of MHC-I to the cell surface (24).

Nef is an intracellular protein that is found both in the cytoplasm and attached to lipid raft membranes via an N-terminal myristoylation signal(1). Previous studies identified several amino acid motifs important for Nef function: an acidic di-leucine motif (ENTSLL160-165) important for CD4 downregulation and viral infectivity; an acidic cluster (EEEE62-65), important for MHC-I downregulation, an N-terminal α-helix, which includes a crucial methionine residue (M20) also required for effects upon MHC-I; and a polyproline helix (PxxPxxPxxP69-78) important for binding interactions with SH3-motifs and possibly involved in MHC-I downregulation (14).

Nef utilizes the components of the endosomal vesicle system to modulate cell surface proteins. The best studied endosomal proteins with which Nef interacts in this system are the clathrin adaptor protein complexes AP-1 and AP-3 (12). Adaptor protein
complexes are part of the cytoplasmic coat of endosomal vesicles. This family of complexes contains four members, and each complex (AP 1-4) consists of four subunits: two large (γ, α, δ, ε and β1-4), one medium (μ1-4) and one small (σ1-4). The AP-2 complex is associated with endocytosis, the AP-1 complex is associated with trafficking between the endolysosomal system and the trans-Golgi network (TGN), while the AP-3 complex is associated with lysosomal trafficking (12). The di-leucine motif of Nef is required for binding to AP-1, -2 and -3 (5). The Nef di-leucine motif mediates interactions with the γ-σ1 and δ-σ3 hemi-complexes of AP-1 and AP-3 (18). While Nef also interacts with the μ1 and μ3, it does not have the canonical tyrosine sorting signal (Yxxφ) usually associated with binding to these subunits. Curiously, the MHC-I cytoplasmic domain (MHC-I CD) contains a tyrosine critical to the effect of Nef on MHC-I downregulation.

In a recent study, siRNA was used to reduce expression of the μ subunit of AP-1, leading to a decrease in Nef-mediated downregulation of MHC-I (23). While the di-leucine motif is necessary for interaction with the adaptor hemi-complexes, it is not required for interaction with the μ1 or μ3 subunits, (12) or for downregulation of MHC-I. A likely scenario drawn from these observations is that the Nef-mediated interaction between AP-1 and MHC-I occurs via the μ1 subunit and is independent of the di-leucine motif. This hypothesis is supported by the observation that the di-leucine motif is not required for recruitment of AP-1 when Nef is co-immunoprecipitated using an antibody against HLA-A2 (23). Nevertheless, the di-leucine motif is required to recruit the intact
AP-1 in GST-pulldown assays (20); it appears that the di-leucine-independent interaction with the μ1 subunit is not sufficient to pull down the intact AP-1 complex.

The hypothesis that interaction with m1 is involved in downregulation of MHC-I by Nef is supported by the existence of a putative tyrosine-based sorting motif in the CD of the HLA-A and B alleles of MHC-I (20). The μ2 subunit of AP-2 has been crystallized while binding the tyrosine motif of the EGF receptor, and it is thought that the μ subunits of other AP complexes are also responsible for binding tyrosine motifs in target proteins. Indeed, the aspartic acid residue (D_{176}) of μ2 required for the stabilization of the tyrosine-based interaction is well conserved in the μ1(D_{174}) and μ3 (D_{182}) subunits, while hydrophobic residues (in μ1, V_{392} and L_{395}) stabilize the interaction of the Y+3 residue in the motif (22). The canonical tyrosine motif contains a hydrophobic residue at the Y+3 position, but MHC-I does not. The fact that the MHC-I CD lacks a hydrophobic residue at the Y+3 position may explain why Nef must be present for MHC-I to interact with AP-1. Indeed, when the Y+3 residue was replaced with a leucine, creating a YSQL sequence, MHC-I was endocytosed in a clathrin-dependent pathway, presumably through interactions with AP-2 (19).

In this study, we explore the hypothesis that the HLA-A2 CD and Nef cooperate to form a novel binding site to AP-1, possibly mediated by the tyrosine-binding pocket of μ1. We also test the hypothesis that residues in Nef involved in MHC-I downregulation would be involved in this novel interaction. Based on data from these studies, a model is offered in which the Nef protein acts as a facilitator of the MHC-I CD/μ1 interaction, resulting in the retention of MHC-I away from the cell surface.
Experimental Procedures

Cell Culture

SupT1 T cells were obtained through the NIH AIDS Research and Reference Reagent Program (Catalog #100, submitted by Dr. James Hoxie), and they were maintained in RPMI medium supplemented with 10% fetal bovine serum, 2mM glutamine, 100 units of penicillin per milliliter (ml), and 100μg of streptomycin per ml.

P4R5 cells have been described previously (7). In brief, they are a HeLa clone expressing both the CD4 receptor and both CXCR4 and CCR5 coreceptors. They were maintained in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100 units of penicillin per ml, and 100μg of streptomycin per ml, and 1μg puromycin per ml.

Plasmids and Constructs

The pBridge (Clontech) and pGEX 4T-1 plasmids containing NL4-3 Nef and NL4-3 Nef LL164-165AA have been described previously (9,17). All other constructs were created using standard PCR mutagenesis and sub-cloning techniques.

Flow Cytometry

SupT1 cells (3x10^6) were transfected during exponential growth with 20 μg of the pCI-neo vector (empty or containing Nef clones) and 2 μg of the pCG-GFP vector (a gift from Dr. Jacek Skowronski) as a transfection marker using the Amaxa Cell Kit V, protocol O-17 (Amaxa Systems, Gaithersburg, MD). Cells were incubated for 24 hours after transfection, then stained with anti-CD4-APC (Becton Dickinson), and anti-HLA-A2 (BB7.1 clone, courtesy of D. Camerini, UC Irvine). Cells were then fixed and analyzed on a Coulter Elite flow cytometer. The average PE and APC fluorescence
intensity of the GFP-positive cells is plotted. Plots shown are representative of multiple experiments.

**GST Pull-downs**

The constructs of the pGEX4T-2 plasmid containing the various proteins were expressed in BL21(DE3) cells. Colonies were inoculated into cultures, and a 25ml culture of LB-Ampicillin (100µg/ml) was grown overnight. The next day, 75ml of LB-Ampicillin was added, and cells were grown for 3-4 hours. Subsequent addition of 1mM IPTG stimulated protein expression, and cultures were grown for 5 additional hours. Cells were washed and aliquotted, and pellets were stored at -80°C until used. For the pull-downs, bacterial pellets were lysed with (50 mM Tris-HCl [pH 8], 5 mM EDTA, 150 mM NaCl, 10mM MgCl$_2$ and 1% Triton X-100, 1mM DTT) for 2 hours, and subsequently incubated with glutathione-labeled beads (GE Healthsciences) for 30’. After 4 washes with cold PBS, aliquots from each preparation were run on an SDS-Page gel (Bio-Rad) and stained with Coomassie (Bio-Rad) to equalize loading of bead-associated proteins into the pull-downs. Cellular lysates were made from P4R5 cells; approximately 2.5x10$^6$ cells were pelleted and then lysed with buffer ((50 mM Tris-HCl [pH 8], 5 mM EDTA, 150 mM NaCl, 10mM MgCl$_2$ and 1% Triton X-100). Lysates were stored at -80°C until used. For pull-downs with cellular lysate, equal amounts of protein-bead complexes were pre-incubated with lysis buffer + 2mg/ml BSA for 30’. One milliliter of cellular lysate was added and incubated overnight. After washes with lysis buffer, protein-bead complexes were processed by western blot, as described below. For pull-downs performed with the isolated µ1 subunit, the Quick Coupled Transcription/Translation Kit
(Promega) was used, utilizing the pTNT vector to express μ1 and the μ1 mutants. The buffers were the same as with cell lysate.

**Western Blot**

Samples for analysis by western blot were taken from the same transfected cells that were analyzed by flow cytometry. Cells were suspended in loading buffer containing SDS and boiled for 10’. After resolution on a 12% denaturing polyacrylamide gel (Bio-Rad), the proteins were transferred to a nitrocellulose membrane and blotted with the following antibodies: anti-γ adaptin (Clone 100/3, 1:1000, Sigma), anti-μ1 adaptin (1:500, a gift from L. Traub, University of Pittsburgh). Detection was performed using a goat anti-mouse antibody linked to horseradish peroxidase (Bio-Rad, Hercules, CA), followed by development with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). Densitometry measurements were taken with the NIH ImageJ program.

**Yeast 2- and 3-Hybrid Assays**

Yeast 2- and 3- Hybrid transformations were performed according to the manufacturer’s instructions (BD Biosciences). Transformants were plated on agar plates containing Leu-/Trp- selective medium. After 5-7 days, colonies were pooled and grown overnight in liquid Leu-/Trp- media. The next morning, 2ml Leu-/Trp- liquid cultures were started at an $OD_{600nm}$ of 0.200, and grown for 4-6 hours to an $OD_{600nm}$ of 0.4-0.6. From this log-phase culture, 5ml of Leu-/Trp- and Leu/Trp-/His- liquid cultures were inoculated at an $OD_{600nm}$ of 0.005. Measurements were taken every 24 hours. Patch plates are pooled colonies from a transformant plate resuspended in 25 μl of Leu-/Trp- media and spotted on Leu-/Trp- plates. The next day patches are replica-plated on Leu-/Trp- and Leu-/Trp-/His- plates. Data shown are representative of 3 separate experiments.
Results

Determinants within Nef involved in downregulation of MHC-I and in vitro binding to AP-1 are poorly correlated.

Nef and AP-1 co-localize in cells, and they directly interact (9,12), yet it is unknown how regions of Nef that are important for MHC-I downregulation affect the AP-1 interaction and intracellular localization. We examined the Nef mutants M20A, E62-65A, P72A-P75A, P78A, and LL164-165AA for MHC-I and CD4 downregulation, and for AP-1 interaction (Figure IV). The M20, E62-65, P72-P75 and P78 residues have been described as having a role in MHC-I downregulation, and these observations are confirmed in flow cytometry of Nef-transfected SupT1 T Cells (Figure IV.1A). As a control for the specificity of the defect of these Nef mutants, CD4 downregulation was also assessed; all mutants defective in MHC-I downregulation appeared functional in regards to CD4 downregulation. The Nef LL164-165AA (Nef LL/AA) mutant had an opposite phenotype; it was able to downregulate MHC-I-A2, but not CD4.

To test interactions with intact AP-1 complexes for these mutants, we utilized a GST pull-down system with cell lysate as a source of AP-1. The Nef LL/AA mutant was significantly defective in the ability to pull down intact AP-1 from cell lysate, corresponding to previous studies (3,11). In contrast, of the Nef residues associated with MHC-I downregulation, only the E62-65A mutant showed a possible defect in the ability to interact with AP-1 although it was not significant (p=0.17). Surprisingly, the M20A showed an increase in the ability to interact with intact AP-1 (p=0.04). The P72A-P75A and P78A mutations appeared to have no detectable effect upon interaction with AP-1.
Figure IV. 1 The Nef-mediated modulation of MHC-I and CD4 in comparison to the interaction with AP-1. Downregulation of MHC-A2 and CD4 and interaction with AP-1 by a panel of Nef mutants. A, SupT1 T cells were transfected with the Nef mutants listed on the left side of the panels and GFP as a transfection marker. Mean fluorescence intensity of GFP+ cells is shown for MHC-I (HLA-A2) and CD4. B, Nef interactions with AP-1 in a GST pull-down system. Underneath is identical membrane stained with Ponceau S to show equal loading of GST-proteins. Densitometry plots of bands were quantified using the ImageJ program. Asterisks indicate significant difference between wtNef and mutants (n=3, student’s t-test, p <0.05).
Thus the interaction between Nef and intact AP-1 does not appear to correlate with MHC-I downregulation, with the exception of the acidic cluster mutant (E$_{62-65}$A).

**The MHC-I CD and Nef together create a unique AP-1 interaction-interface independent of the Nef di-leucine motif.**

A goal of this project was to understand the role of Nef motifs in the MHC-I/Nef/AP-1 interaction. To that end, I first attempted to utilize a yeast 3-hybrid system to examine binding between Nef, the MHC-I-A2 CD, and µ1, which we hypothesized was the likeliest AP-1 subunit for this interaction. However, various combinations of Nef, µ1, and MHC-I-A2 CD in this system each failed to give a positive interaction. The direct binding between Nef and the MHC-I CD was characterized in a previous study as a weak interaction, one that required cross-linking by DSP for detection in a GST pull-down (27). The negative results of the yeast 3-hybrid experiments were likely a consequence of the weakness of this interaction. It is also probable that in the GST pull-down studies performed in Figure I.B, any interaction mediated by the presence of MHC-I in the cell lysate would be too weak to detect.

A strategy to bypass the requirement for MHC-I and Nef interaction was formulated after the publication of a study that used a chimera of MHC-I-A2 and Nef (23). Following this lead, a fusion protein was created between the MHC-I-A2 CD to the N-terminus of Nef ("A2_Nef", Fig IV.2A), and this chimera was first utilized in experiments using the GST pull-down method with cell lysate. The Nef/MHC-I CD interaction in vivo is likely facilitated by the membrane-localization of Nef and MHC-I to membranes, which are disrupted in the in vitro assays. This fusion protein enabled the
Figure IV.2 The MHC-I-A2 Cytoplasmic domain and Nef co-operate in a synergistic interaction with the AP-1 complex. A, model of the GST-A2_Nef chimera, a fusion protein created between the MHC-I-A2 cytoplasmic domain (“HLA-A2 tail”) joined to the N-terminus of the NL4-3 Nef protein. Important residues within the MHC-I-A2 CD and Nef are indicated; drawing is not to scale. B, GST pull-down of AP-1 complexes from HeLa cell lysate, performed as in Figure 1. The synergistic interaction between Nef and the A2-CD is seen in the fusion protein, (“A2_Nef”). This interaction is Nef di-leucine independent, but dependent on the Y_{320} residue of MHC-I A2.
examination of interactions between Nef, MHC-I, and AP-1 that were not dependent upon Nef and MHC-I binding.

In the GST-pulldown system with intact AP-1 complexes, the MHC-I-A2 CD alone did not detectably interact with AP-1 (“A2”, Figure IV.2B). However, when fused to the N-terminus of Nef, the MHC-I-A2 CD induced a synergistic interaction with AP-1 that did not require the Nef di-leucine motif (Figure IV.2B, compare NefLL/AA with A2_Nef LL/AA). While independent of the canonical Nef AP-interaction motif, the A2-mediated interaction did require the Y_{320} residue of the HLA-A2 CD. This residue has been previously shown to be necessary for the effect of Nef on MHC-I, and thus it appears that the AP-1/A2_Nef LL/AA interaction correlates with this determinant of the MHC-I downregulation phenotype.

The MHC-I CD-dependent A2_Nef/AP-1 interaction requires the acidic cluster and P_{78} of Nef.

After establishing that the interaction between A2_Nef and AP-1 is independent of the Nef di-leucine motif, we sought to address which regions of Nef were involved. Of those tested, the M_{20} residue, EEEE_{62-65}, P_{72}-P_{75}, and P_{78} contribute to downregulation of MHC-I (Figure IV.1A). The MHC-I CD-dependent and Nef di-leucine independent interaction of A2_Nef with intact AP-1 complexes required both the acidic cluster (EEE_{62-65}) and the P_{78} residues. However, the M_{20} or P_{72}-P_{75} residues were dispensible (Figure IV.3). The lack of a role for P_{72}-P_{75} or M_{20} in AP-1 interaction will be addressed in the discussion.
Figure IV.3 Residues of Nef involved in the di-leucine independent synergistic interaction with AP-1. The GST pull-down system used in Figures IV.1 and 2 was used to study the roles of Nef residues previously determined to be important in MHC-I downregulation. In addition to blotting for γ-adaptin, a Western blot for the μ1 subunit is also shown; the two subunits clearly precipitate together. A Ponceau S stain of the identical membrane is shown as a loading control for GST-proteins.
The interaction of A2_Nef and AP-1 is mediated by the μ1 subunit, but not the γ-σ1 hemi-complex of AP-1

As previously noted, Nef interacts with the AP-1 complex via di-leucine-mediated binding to the γ-σ1 hemi-complex and via di-leucine independent binding to the μ1 subunit. Since downregulation of MHC-I is also independent of the Nef di-leucine motif, we hypothesized that the AP-1 subunit relevant to the A2_Nef interaction is μ1.

Two different experimental methods were used to test this hypothesis. GST pull-down studies were performed with in vitro translated μ1 instead of intact AP-1 complexes from cell lysates. The results with μ1 in isolation recapitulated those found with intact AP-1 complexes; there was a synergistic increase in μ1 binding by cooperation of Nef and the MHC-I CD (Figure IV.4A). Additionally, this interaction was dependent upon the Y320 of the MHC-I CD. In the yeast 2-hybrid system, the A2_Nef chimera showed an increase in avidity compared to Nef alone (Figure IV.4B). This was also dependent upon the Y320 of the MHC-I CD, in a manner consistent with the MHC-I CD-dependent interaction with μ1 and AP-1 (Figure IV.4A, Figure IV.2). To confirm that the interaction was limited to the μ1 subunit, the yeast 3-hybrid system was used to examine binding to γ-σ1 hemi-complex (Figure IV.C), which is dependent upon the Nef di-leucine motif. The addition of the MHC-I CD to Nef did not confer a di-leucine independent ability to interact with γ-σ1, nor did it enhance Nef interaction when the di-leucine motif was intact. Thus, the μ1 subunit is responsible for the synergistic interaction between Nef, the MHC-I CD, and the AP-1 complex.
Figure IV.4. The µ1 subunit of AP-1 is responsible for the A2_Nef di-leucine independent interaction. A, Pull-downs were performed using S\textsuperscript{35}-methionine labeled µ1. The gels were stained with Coomassie Blue prior to exposure to film to control for loading. B, In the yeast 2-hybrid system, the Nef:µ1 interaction is less avid than the A2_Nef:µ1 interaction. The increase mediated by the MHC-I-A2 CD is dependent upon Y\textsubscript{320}. Interactions are measured as growth in selective media over time. C, In the yeast 3-hybrid system, the A2_Nef chimera is unable to interact with the γ–σ1 hemi-complex in the absence of the Nef di-leucine motif, and the MHC-I-A2 CD provides no additional affinity compared to Nef alone.
The acidic cluster of Nef is involved in binding to μ1.

To further examine correlations between the A2_Nef/μ1 interaction and downregulation of MHC-I, mutations in regions of Nef important in downregulating MHC-I were examined for binding to μ1 (Figure IV.5). As in interaction with intact AP-1, the M20 and polyproline helix (P72-P75) were not required in MHC-I CD/Nef/μ1 interaction. Importantly, the acidic cluster of Nef was directly involved in binding to μ1 with and without the MHC-I CD. This result provides a direct role for this motif in binding to AP-1, rather than an indirect role via PACS-1, as suggested previously. Curiously, although the P78 residue was required in the di-leucine independent interaction with AP-1, it did not appear to be required for binding to the μ1 subunit. Despite the appearance of a less intense μ1 signal in the A2_P78A_LL/AA lane of Figure V, the corresponding Coomassie stain indicates that GST-A2_P78A_LL/AA was underloaded, and is therefore attributable for the lower intensity of the μ1 signal. Subsequent experiments have confirmed this finding (data not shown). The discordance between the role of P78 in MHC-I CD-dependent interaction with AP-1, but not in interaction with μ1, will be addressed in the discussion.

The Nef/MHC-I CD/AP-1 interaction may occur in the tyrosine-binding pocket of μ1.

Studies on the regions of μ1 involved in this interaction provided insight into the possible mechanism of binding. The tyrosine-motif-binding pocket of the μ subunits has been crystallized with a canonical YxxL motif from EGFR (22). Contact points between the tyrosine residue and μ2 include LFLD176, K203, and V402/L405. These
Figure IV.5. The acidic cluster of Nef is responsible for interaction with μ1. The pull-down system was used to assess the roles of Nef residues in the chimera di-leucine independent interaction with S$^{35}$-labelled μ1. The left hand panels are GST-Nefs alone; the right-hand panels are in the context of the A2_Nef LL/AA chimera. Bottom panels are corresponding gels stained with Coomassie Blue.
residues are conserved between μ subunits, and enabled corresponding mutations to be made in μ1 to examine the role of the tyrosine-motif-binding pocket in the Nef/MHC-I/μ1 complex. The μ1 D174A and μ1 V392A/L395A mutants were expressed in the in vitro translation system, and tested for defects in binding to A2_Nef (Figure IV.6). Both mutants were expressed poorly in comparison to wild type μ1. Nonetheless, the results were interpretable. Surprisingly, the μ1 D174A mutation did not eliminate the synergistic interaction; a possible explanation for this is offered in the discussion. The μ1 V392A/L395A mutations appeared to negate the increase in the binding to μ1 conferred by the MHC-I CD. Based on this data, the hydrophobic-binding pocket of the μ1 subunit is likely involved in this trimolecular interaction.

A mutation toward a canonical tyrosine motif in the MHC-I tail rescues Nef-mutants defective for interaction with AP-1.

As the MHC-I CD lacks a canonical tyrosine-based sorting signal (Yxxφ), we hypothesized that Nef compensates for the missing Y+3 hydrophobic residue, enabling an interaction with μ1. Indeed, in the context of the A2_Nef chimera, the MHC-I Y320 appears to act in a fashion similar to a Yxxφ motif, in that it mediates an interaction with μ1. To extend this model, the putative sorting signal (YSQA) in the MHC-I CD was mutated to resemble a canonical sorting signal (YSQL). The MHC-I-A2 YSQL mutant did not interact with AP-1 (Figure IV.7A). However, changing the YSQA to YSQL was able to rescue the binding of the A2_Nef E62-65A_LL/AA and A2_P78A_LL/AA chimeras (Figure IV.7B), which were defective in the YSQA context. These data provide further evidence for a role of the hydrophobic binding pocket of μ1 in the trimolecular
Figure IV.6. Mutations in the tyrosine-binding pocket of μ1 and their effect upon the co-operative interaction between MHC-I, Nef, and μ1. A, GST pull-downs were performed simultaneously with duplicate sets of bait, GST-A2_Nef LL/AA. Top panel was incubated with wt μ1, bottom panel with μ1 D174A. B, Experiment was performed exactly as in A, except the second set of bait was incubated with μ1 VL392/395AA.
Figure IV.7 A canonical sorting signal in the MHC-I-A2 CD rescues the defective binding of Nef mutants to AP-1. A, GST pull-downs of intact AP-1 complexes using a wild type and modified MHC-I-CD as bait (A2_YSQA, A2_YSQL) are unable to interact with AP-1 in the absence of Nef. B, The YSQL motif is able to rescue AP-1 binding of A2_Nef E62_65A_LL/AA mutants or A2_NefP78A_LL/AA.
interaction of Nef, the MHC-I CD, and AP-1. The data also suggest a functional
equivalency between the engineered L323 residue in the MHC-I-A2 CD and the EEEE$_{62}$-
$P_{78}$ regions of Nef. Finally, the data suggest that other, as yet identified, residues of Nef
are required to allow even the YSQL sequence to bind to AP-1.
Discussion

In this study we sought to illuminate the mechanism by which HIV-1 Nef, the AP-1 complex, and the MHC-I CD interact. While previous studies have described a role for AP-1 in Nef-mediated downregulation of MHC-I, those findings have been extended here by careful analysis of the protein-protein interactions within the trimolecular complex.

Initial studies examined the regions of Nef required for the downregulation of MHC-I and evaluated their roles in the AP-1 interaction. The only motif that appeared required for both functions was the acidic cluster (EEEE\textsubscript{62-65}). This was not an entirely unexpected finding, as a previous model proposed binding via EEEE\textsubscript{62-65} to PACS-1, which would connect Nef to AP-1. To better understand interactions with AP-1 relevant to MHC-I downregulation by Nef, Nef was joined to the cytoplasmic domain of MHC-I-A2, and used in further studies. Strikingly, a novel, cooperative binding to AP-1 and \( \mu_1 \) was discovered in using this MHC-I CD/Nef chimera. This cooperative interaction was independent of the Nef di-leucine motif, suggesting that it was correlative to the AP-1 dependent portion of Nef-mediated downregulation of MHC-I. Further evidence in support of this hypothesis came from studies mutating residues of Nef associated with MHC-I downregulation; the acidic cluster and proline 78, both involved in modulation of MHC-I in vivo, were required for the cooperative interaction between Nef, MHC-I, and AP-1. Subsequent studies establishing the \( \mu \) subunit of AP-1 as the target of this interaction, along with the evidence that the tyrosine of the MHC-I CD was also required,
led to the idea that the mechanism of binding occurs in a manner similar to that between μ subunits and Yxxφ motifs.

However, as the YSQA sequence in the MHC-I CD does not conform to the canonical tyrosine-sorting signal, it requires a cooperative interaction via Nef to supplement the binding to μ1. In the search for elements of Nef involved in this interaction, it was discovered that the Nef acidic cluster was capable of mediating binding to μ1 in the absence of the MHC-I CD, and that this motif was required for m1-binding even in the presence of the MHC-I CD. It seems that the Nef EEEE$_{62-65}$ motif binds μ1 independently of the MHC-I CD; this is highly interesting, as this is the first known instance of an acidic cluster motif being implicated in direct binding to μ1. Additionally, these data provide strong evidence for a Nef/AP-1 interaction that is independent of PACS-1.

Circumstantial evidence for acidic cluster motifs mediating interactions with AP-1 comes from recent studies of the mannose 6-phosphate receptor (MPR) family. Using surface plasmon resonance with purified AP-1, an acidic cluster (EESEE) of the cation-dependent mannose 6-phosphate receptor (CD-MPR) cytoplasmic domain was determined to be involved in interaction with AP-1 (25). While this motif can be phosphorylated by casein kinase II (CKII), this phosphorylation was not required for binding, an interesting point in consideration of the fact that Nef’s acidic cluster is not phosphorylated by CKII. Another acidic cluster motif involved in AP interaction is found in the cation-independent mannose 6-phosphate receptor (CI-MPR); however, these interactions were highly dependent upon phosphorylation on both the acidic cluster CKII
site and the AP-1 complex itself (13). A possible mechanism for the direct binding may be that the acidic cluster of Nef, CD-MPR or CI-MPR interact with \( \mu \) through ionic bonds between the negatively charged acidic residues and the positively charged surface of the \( \mu \) subunits (23). Phosphorylation of CKII sites would increase the overall negative charge, possibly enhancing the interaction.

While ionic interactions with the surface of \( \mu 1 \) may be responsible for interaction with the E62-65 residues of Nef, the region of \( \mu \) binding by Y\(_{320} \) of the MHC-I CD is most likely the tyrosine-binding pocket. This region includes the residues LFLD\(_{174} \), K\(_{201} \), and V\(_{392}\)L\(_{395} \). Interestingly, mutations within the hydrophobic pocket of this region (VL\(_{392}/395\)AA) diminished the co-operative MHC-I-A2 CD/Nef interaction. However, mutation of a residue (D\(_{174} \)A) predicted to interact with the tyrosine residue of YxxL motifs did not affect the interaction. It is possible that other residues of the tyrosine-binding-pocket may be involved in Nef/MHC-I/\( \mu 1 \) binding. In a study characterizing interactions of the SDYQRL motif of the trans-Golgi network resident protein 38 (TGN38) cytosolic domain with \( \mu 1 \), both the \( \mu 1 \) D\(_{174} \) and F\(_{172} \) residues were mutated to abrogate binding (16). Thus, future work may utilize a double mutant of \( \mu 1 \), with both D\(_{174} \) and F\(_{172} \) changed to alanine.

Alternatively, in a comparable study of tyrosine-motif interactions with the \( \mu 2 \) subunit, a D\(_{176} \)A mutation was sufficient to abolish binding to the SDYQRL motif of TGN38. However, binding of \( \mu 1 \) D\(_{176} \)A to the cytoplasmic domain of EGFR remained intact (21). A possible explanation for the differences between TGN38 and EGFR is that three sorting signal domains have been described for EGFR, including a PxxP motif, a YxxL motif, and a LL motif (6,26). It is conceivable that the PxxP or LL motifs bind
outside the tyrosine-binding pocket, supplementing the YxxL-based interaction with μ2.

In a similar fashion, P$_{78}$ of Nef may supplement a tyrosine-based interaction between the MHC-I CD and AP-1.

Evidence in support of this hypothesis comes from a series of experiments in which the YSQ$_{A}$ sequence in the MHC-I-A2 CD was changed to YSQL, creating a canonical tyrosine motif. Importantly, creating a YxxL interaction motif on the MHC-I-A2 tail did not increase the AP-1 binding in the context of the A2$_{-}$Nef LL/AA chimera; this suggests that these contacts use similar mechanisms, i.e. interaction with the μ1 subunit. While, the YSQL motif was not sufficient to interact with AP-1 in isolation, it enabled an interaction in the context of A2$_{-}$Nef chimeras defective for interaction with AP-1. Specifically, the YSQL mutation rescued defects caused by mutating Nef E$_{62-65}$ or P$_{78}$; these data suggest that other, unknown residues in Nef may contribute to interaction with AP-1.

In the initial study describing the Nef/MHC-I/AP-1 interaction suggested roles for Y$_{320}$ of MHC-I, and the N-terminal α helix of Nef, but not for the acidic cluster or P$_{78}$ (23). In that study, the acidic cluster is proposed to interact with the MHC-I CD. Those results differ from the data presented here; to resolve this discordance, it is important to consider the context of the previous mutational analysis. The Nef di-leucine motif was intact in the acidic cluster and polyproline helix mutants, and its interaction with AP-1 could overcome any defects exhibited by the acidic cluster or P$_{78}$ mutants. Yet the N-terminal mutants of Nef were defective in the AP-1 co-immunoprecipitation assay even in the presence of an intact di-leucine complex. An alternative explanation is that differences between the methods of co-immunoprecipitation from cells versus GST-
mediated precipitation from cell lysates may reveal differences in Nef/AP-1 interactions. For example, the Nef di-leucine dependent and MHC-I CD-dependent interactions with AP-1 appeared synergistic in the GST pull-downs presented in this study (Figure IV.2, binding affinities for AP-1: A2_Nef > A2_Nef LL/AA = A2_Y320A_Nef). Yet in the previous study, no difference is seen in the co-immunoprecipitation of AP-1 with or without the Nef di-leucine motif ((23) Figure 6A, binding affinity for AP-1: A2/Nef = A2/LL164-165AA). It is possible that the MHC-I/Nef/AP-1 interaction has several conformations dependent upon its intracellular localization, and that the co-immunoprecipitation is selecting for only one of the conformations.

In support of the in vivo relevance of the synergistic ability of the MHC-I CD and Nef to bind AP-1, two motifs associated with MHC-I downregulation were important for this interaction: the acidic cluster (EEEE62-65), and the last proline of the polyproline helix (P78). Curiously, mutations affecting the N-terminal α-helix (M20A) and the SH3-binding domain (P72A-P75A) did not inhibit binding to AP-1. It is possible that these motifs are involved in binding to the MHC-I CD itself, which would be unnecessary in the context of the fusion protein. Alternatively, the polyproline helix might be involved in a step of MHC-I downregulation that does not involve AP-1, for example in increasing endocytosis by ARF6 activation (2).

In summary, these studies have served to further illuminate the mechanism by which HIV-1 Nef downregulates MHC-I. The Nef acidic cluster is proposed to act as a direct contact surface with the μ1 subunit of AP-1. This interaction could be flexible in its requirements; as long as a threshold of negative ionic charge were generated and
presented, it might not demand specific constituents. This idea is supported by the modest
diversity of the Nef acidic cluster; in Chapter II, multiple insertions can be seen in the
Nef acidic clusters of primary clones. The mechanistic role of proline78 is harder to
imagine, but the fact that it plays a part in the chimera/μ1 interaction extends the
understanding of this residue, which is currently limited to the knowledge that it may be
involved in limiting the recycling of MHC-I (4). Future experiments begun in our lab
hope to clarify and extend the understanding of the nature of this interaction by pursuing
a crystal structure of the A2_Nef/μ1 complex.

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Chapter V: Conclusion
Summary and Implications

The objective of this dissertation was to examine in vivo the importance of immune evasion by HIV-1 Nef via MHC-I downregulation, and to extend the knowledge of the mechanism behind this function. By evaluating Nef clones from clinical samples, and by examining protein interactions through in vitro assays, it is hoped that the objective has been met.

MHC-I downregulation by Nef is conserved across sexual transmission.

The Nef protein performs multiple important functions for HIV. Nef enhances infectivity and replication, which helps the virus establish and maintain itself. Nef is also a primary factor in the ability of HIV to evade the host immune system. Nef reduces the amount of MHC-I and MHC-II molecules at the cell surface, reducing immune-based interactions dependent upon CD4+ and CD8+ T cells. Additionally, Nef is the primary target of the early CTL response, and the ability of Nef to tolerate mutations associated with CTL escape is another way in which this protein contributes to immune evasion.

The functions of Nef are to an extent genetically separable, an initial hypothesis stated that replication would be optimized immediately after infection in a naïve host even at a cost to fitness of immune evasion functions. To test this idea, six transmission pairs were identified in which the recipients were acutely infected and possibly immune naïve. Clones of nef were isolated from individuals in the transmission pairs and later tested for functions related to replication and immune evasion.

The presence of diversification of nef sequences after the transmission branch, as well as the evidence for selective pressures occurring within sequences from both sources and recipients, suggested that there was an opportunity for optimization of Nef function
by adaptive evolution. To test if these adaptive changes led to a changed in function, Nef clones were expressed in SupT1 T cells by transient transfection. Transfected cells were measured for downregulation of CD4 (a correlate for enhancement of replication) and for downregulation of MHC-I (correlating to immune evasion). In both sources and recipients, the majority of clones assayed, including the representative sequence from each individual, were completely functional for both CD4 and MHC-I downregulation. The conclusion drawn from these data is that both functions were preserved across sexual transmission despite mutations associated with pressure by CTL.

A possible explanation for the maintenance of both functions after transmission is that Nef-specific CTL were detected even in the most acutely infected of the individuals, and therefore the window for sampling Nef clones optimized for infectivity over immune evasion may be relegated to a very narrow time period. Additionally, the mutations that would disrupt function could have been selected against before they became established in the population; isolated dysfunctional Nef clones were found in three individuals. Another interesting observation in support of selection to preserve function is that despite changes induced by selective pressures, residues associated with Nef functions were unchanged or had conservative mutations. For example, the E and LL of the C-terminal acidic di-leucine motif associated with CD4 downregulation were universally conserved. The methionine associated with the N-terminal α-helix involved in MHC-I downregulation either remained constant throughout the clonal population, or in one pair, was conservatively replaced by an isoleucine. Furthermore, the acidic cluster associated with MHC-I downregulation remained functional even with minor variability between Nef clones, and the polyproline helix was intact in the entire cohort. These data suggest
that in addition to functions associated with CD4 downregulation, MHC-I downregulation is important in the earliest stages of infection in vivo.

**Nef downregulates MHC-I by enabling linkage to the endosomal system via the µ subunit of AP-1.**

The importance of MHC-I downregulation in vivo confirmed the need for studies examining the mechanism behind this function. Interactions between Nef and the AP complexes have been studied for some time, with the central focus upon the canonical AP-binding motif in Nef, the di-leucine motif. However, while the di-leucine motif can account for binding to the γ−σ1, α−σ2, and δ−σ3 hemi-complexes of adaptors, it is not associated with binding µ subunits (1). The di-leucine motif is also involved in downregulating CD4, and it is possible that a hemi-complex interaction is involved in modulating CD4 trafficking. What, then, could be the physiological correlate to µ binding?

A hint to the answer to this question came from the observation that Nef and AP-1 colocalized in a juxtanuclear area that appeared to coincide with the same area in which Nef and MHC-I were found. Peculiarly, the best-known AP-1 interaction motif in Nef is the di-leucine motif, which is dispensable for MHC-I downregulation. An important link to help resolve this paradox came from of a study published by the Collins lab in 2004, showing co-immunoprecipitation of Nef, MHC-I, and AP-1 even in the absence of the Nef di-leucine motif (2). Curiously, MHC-I does not interact with AP-1 in the absence of Nef. It appeared that in the context of the Nef/MHC-I complex, a novel contact interface with the AP complex was revealed.
I extended these studies to understand the nature of this novel interaction, as described in chapter IV. GST pull-downs showed that: 1. Nef, when fused to the MHC-I tail, bound more avidly to intact AP-1 complexes. 2. This increase was dependent upon the tyrosine of the MHC-I tail, and the acidic cluster and P78 residues of Nef, but not the Nef di-leucine motif. 3. The μ1 subunit could recapitulate the pattern of interaction with the intact AP-1 complex. These studies led us to the hypothesis that a Nef/MHC-I/μ1 interaction was responsible for redirecting MHC-I from its normal pathway to an intracellular region, thus preventing presentation of epitopes to CTL.

These data led to the next series of experiments, which were intended to provide in vivo relevance to the MHC-I CD/Nef chimera by asking whether this fusion construct yielded a sorting signal recognized in live cells. Based upon a previously characterized CD8-fusion system, in which the protein of interest is connected to the membrane by covalent linkage to the CD8 extracellular and transmembrane domains (3), I examined the intracellular localization of the Nef and A2_Nef chimeras in comparison to AP-1 (Figure V.1). As previously shown, the CD8-Nef chimera localized to an internal compartment, while mutation of the di-leucine motif in Nef (CD8-Nef LL/AA) led to cell surface localization. CD8 fused to the MHC-I-A2 CD alone (CD8-A2) was localized to the cell periphery. Surprisingly, fusing the MHC-I-A2 CD to Nef LL/AA (CD8-A2_Nef LL/AA) did not confer the ability to localize to an intracellular compartment. It would appear that internalization is dependent upon the Nef di-leucine motif. What could account for the inability of the A2_NefLL/AA sequence to direct internalization? It is possible that the purpose of the MHC-I/Nef/AP-1 interaction is not to prevent the protein
Figure V.1 The intracellular localization of the A2_Nef chimera is dependent upon the Nef di-leucine motif. The Nef and A2_Nef proteins utilized in Chapter IV were fused to the extracellular and transmembrane domains of CD8 in order to study intracellular localization.
from reaching the cell surface, but instead becomes relevant once MHC-I has been internalized. This internalization could be mediated via a non-clathrin pathway such as the ARF6 endosomal system. Once internalized, the Nef/AP-1/MHC-I CD interaction could be involved in blocking recycling from endosomes, a more likely place for AP-1 to be present than the cell surface. If this theory is validated, it might explain why $P_{78}$, but not $P_{72}$ or $P_{75}$ of Nef is involved with the AP-1 interaction, as only $P_{78}$ is proposed to have a role in blocking recycling of MHC-I.

The basic requirements of the Nef/AP-1/MHC-I CD complex include the $\mu_1$ subunit of AP-1, the Y$_{320}$ of the MHC-I CD, and the acidic cluster and $P_{78}$ of Nef. Curiously, $P_{78}$ of Nef is unnecessary for $\mu_1$ binding, and might be involved in interaction with another portion of AP-1, i.e. the $\beta$ subunit. Within $\mu_1$, the determinants involved in the MHC-I CD/Nef interaction include the tyrosine-binding pocket, specifically V$_{392}$/L$_{395}$.

**A model of the MHC-I/Nef/\(\mu_1\) interaction**

In summary, a model is proposed wherein the tyrosine of the MHC-I-A2 CD interacts within the $\mu_1$ tyrosine-binding pocket in the presence of Nef (Figure V.2). The acidic cluster of Nef is suggested to interact with the positively charged surface of $\mu_1$, stabilizing the binding of Y$_{320}$ to the pocket. The N-terminal $\alpha$-helix is proposed to directly bind to the MHC-I CD, explaining why the M$_{20}$ residue would not be required for the AP-1 interaction in the context of the A2_Nef chimera. Additionally, note that the di-leucine motif itself, which would be localized at the bottom of the Nef molecule farthest from the $\mu$ subunit, would be exposed and able to interact with the $\gamma$–$\sigma$1 hemi-complex.
Figure V.2. A model of the Nef/MHC-I CD/µ1 interaction. The protein structures are based upon NMR and crystal studies with Nef and µ2 bound to the P-selectin cytoplasmic domain; this is only a model, and is not based upon structural studies of the actual Nef:MHC-I CD:µ1 complex. Nef is in yellow, the MHC-I-A2 CD is in green, and basic residues of the µ1 subunit are in blue. The amino acids proposed to be involved in the interaction are indicated; the µ1 tyrosine binding pocket residues are orange, the cytoplasmic domain of MHC-I-A2 is in green with Y320 in purple, the proline 78 of Nef is in green, and the acidic cluster of Nef is in red.
This model conforms to the data in Chapter IV, and it will be very interesting to see how it compares to a crystal structure of the MHC-I-A2 CD_Nef:μ1 complex.

**Conclusion**

The work presented here was focused upon the ability of HIV-1 Nef to enable immune evasion, a critical step in successful infection. Specifically, this body of work provides both an in vivo context for the function of MHC-I downregulation by HIV Nef, and an examination of the cellular mechanism behind this function. Subsequent studies may examine how Nef amino acid variations present in the transmission cohort might affect AP-1 interaction. A collaboration is currently in progress to produce a crystal structure of the Nef/MHC-I/AP-1 complex. The points of interaction would be confirmed, and a better idea of where, precisely, in the μ1 subunit this interaction is taking place can be obtained. The unique nature of this interaction lends itself well as a target for new drug therapies; AP-1 and MHC-I only interact when Nef is present.

The importance of continuing research on HIV cannot be overstated. One need only refer to the rising death toll, number of orphans created by AIDS, and increase of drug-resistant strains to realize how dire the situation truly is. Exploration for new drug targets and more efficacious vaccines begins at the level of basic science, and with these data it is hoped that some contribution has been made towards a better understanding of Nef evolution and interactions that can be utilized to make a better weapon against HIV.
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