

**NEF COMMANDEERS HOST CELLULAR FACTORS
TO PROMOTE HIV-1 IMMUNE EVASION**

by

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ABSTRACT

Despite mounting an active immune defense, the Human Immunodeficiency Virus (HIV) persists as a low-level infection in the blood in infected individuals. The HIV protein, Nef, is indispensable for disease progression and promotes viral spread by allowing HIV infected cells to evade recognition by the host's immune system. Cytotoxic T Lymphocytes (CTLs) recognize and lyse cells that present viral antigens in complex with class I-major histocompatibility complex (MHC-I) molecules. Nef directly interacts with, downmodulates, and degrades MHC-I molecules, thus rescuing infected cells from CTL recognition and subsequent death.

Nef binds directly to the cytoplasmic tail of specific MHC-I molecules and recruits the clathrin-associated adaptor protein AP-1 to redirect MHC-I from the plasma membrane to the *trans*-Golgi to endosome trafficking loop. To better understand the mechanism by which Nef promotes HIV immune evasion, we performed a comprehensive screen of domains in the HLA-A2 cytoplasmic tail (Y₃₂₀SQA₃₂₃ASSD₃₂₇), Nef (M₂₀, E₆₂₋₆₅, and P_{72/75/78}), and AP-1 (FD_{172/174}) and determined that amino acids in each of these domains are required for HLA-A2 downmodulation and formation of the Nef-MHC-I-AP-1 complex. Importantly, we described key amino acids in the cytoplasmic tail of HLA-A2 that explain why Nef can downmodulate HLA-A and HLA-B, but not the natural killer (NK) cell inhibitory molecules HLA-C or HLA-E. Thus Nef allows infected cells to evade lysis by both CTL and NK cells. Furthermore, we explored the mechanisms of Nef-dependent recruitment of AP-1. We determined that Nef requires functional ARF-1, an AP-1-scaffold protein, to recruit AP-1 to the cytoplasmic tail of MHC-I.

Additionally, Nef downmodulates the viral co-receptor CD4 to promote HIV assembly and release by accelerating its endocytosis. The CD4 pathway appears to be distinct from how Nef reroutes MHC-I from the *trans*-Golgi. However, these disparate pathways ultimately converged in a COP-I coatomer β -COP-dependent degradative

pathway. Overall, our findings reveal the mechanism by which Nef suppresses recognition of HIV-infected cells and limits clearance by the host's immune system.

CHAPTER I

Introduction to HIV-1 and Immune Evasion

Overview

My dissertation focuses on the mechanism by which HIV-1 escapes recognition by the host immune system. The first chapter reviews research performed in the HIV-1 field on immune evasion that guided the development of my research hypothesis found in the subsequent chapters of this dissertation. Chapter I will be submitted as a requested review article in the journal *Advances in Viral Research*. Chapter II was published in the *Journal of Biological Chemistry* and details the domains required for a three-way complex between MHC-I, AP-1, and Nef. Chapter III was published in the *Public Library of Sciences: Pathogens* online journal and describes two distinct Nef-induced trafficking pathways that converge to enhance degradation of MHC-I and CD4. Malinda Schaefer was first author on this paper. Because the project was extensive, I had significant contributions to the conclusions in this composition leading to my position as second author. Chapter IV will be submitted as my second first-author manuscript for peer review and implicates the GTPase ARF-1 in Nef-induced AP-1 recruitment and downmodulation of MHC-I molecules.

HIV-1 Pathogenesis

Despite major advances in research and treatment, the human immunodeficiency virus (HIV) continues to persist as a pandemic. 33 million people are currently living with HIV, and in the last year approximately 2 million people have died of acquired immunodeficiency syndrome (AIDS). HIV/AIDS has started to reverse advancements in

life expectancy in Africa (1). An effective, prophylactic vaccine has proven elusive, and a cure seems even more distant.

Following initial infection, the individual's immune system mounts an active defense against the virus (2,3). During the acute phase, anti-HIV cytotoxic T lymphocytes (CTLs) and antibodies are generated (4). Throughout the course of disease, HIV has been shown to preferentially infect and destroy HIV-specific CD4⁺ T lymphocytes, thus accounting for a loss of anti-HIV immune response (5). During latency the number of virus copies per mL of blood (plasma viral load) correlates with the rate of onset of AIDS and eventual death (6). Once the CD4⁺ T cell count reaches <200 cells per μ L of blood, the immune system is considered functionally impaired, the clinical definition of AIDS, and opportunistic infections can take hold in HIV infected individuals.

HIV-1 Genome and Replication

The Baltimore virus classification system sorts HIV-1 into group VI. This classification into the family retroviridae reflects that HIV-1 reverse transcribes its single-stranded RNA (ssRNA) genome into a DNA intermediate prior to integrating into the host cell DNA. HIV-1 is further categorized into the genus lentivirus, which is typically characterized by a chronic infection with a latent incubation period.

As seen in Figure 1.1, the viral lifecycle starts with the HIV-1 particle binding to the combination of CD4 and either of two chemokine receptors, CCR5 or CXCR4. Upon membrane fusion and viral entry, the viral core is released into the cytoplasm of the infected cell. Reverse transcription takes place in the cytoplasm using the ssRNA template to make one viral dsDNA genome. Bound by the pre-integration complex, the viral DNA is transported into the nucleus and integrated into the host genome. The viral genome is now called the provirus and is the template for transcription of viral RNAs. After transport out of the nucleus, viral RNAs are translated by host ribosomes to make viral proteins which are trafficked to and assemble at the plasma membrane. Two full length ssRNA genomic transcripts are recruited into the budding virus, the envelope membrane pinches off, and the viral particle is released and matures (reviewed in (7)).

As seen in Figure 1.2, the HIV-1 genome contains three canonical genes that retroviruses encode: group antigens (*gag*), polymerase (*pol*), and envelope (*env*). Gag is produced as a 55 kilodalton structural protein that is necessary and sufficient for viral-like particle assembly and release in the absence of viral genome or other viral proteins (8). Upon budding and release from the infected cell, Gag is processed by HIV-1 protease into four mature proteins: p17 matrix, p24 capsid, p7 nucleocapsid, and p6 (reviewed in (7)). The *pol* gene encodes viral enzymes, including protease, reverse transcriptase, and integrase. Likewise, Env is produced as a 160 kilodalton protein that is processed into the surface protein, gp120, and the transmembrane protein gp41. gp120 is responsible for viral entry into cells because it directly interacts with the viral co-receptor CD4 (9). Viruses have long been shown to downmodulate their entry receptor to prevent superinfection by viruses using the same receptor (10). HIV-1 Env has been shown to interact with and reduce the surface expression of the viral co-receptor CD4 (11) in order to prevent viral superinfection (12) and enable viral particle assembly and release (13,14).

Also shown in Figure 1.2 are *tat* and *rev* which encode important regulatory proteins for HIV-1 expression. Tat binds to the *trans*-activating response element (TAR) in the LTR to promote transcription elongation (15). Rev binds to Rev Response Elements (RRE) in viral RNA transcripts to promote nuclear export and RNA stability and utilization (16).

Unlike other retroviruses, HIV-1 has acquired accessory genes, *vif*, *vpr*, *vpu*, and *nef*, which encode proteins that optimize viral fitness and spread. The Vif protein degrades the cellular cytidine deaminase, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), which has been shown to hypermutate the viral genome during reverse transcription, thus causing production of non-infectious virus (17). Incorporated into the virion (18,19) and pre-integration complex, Vpr causes infected cells to undergo a G₂ mitotic arrest (20), a state which favors transcription from the HIV-1 LTR (21). Vpu also downmodulates the immune receptor CD4, and recently has been shown to combat an anti-viral-budding protein, tetherin (22).

Finally, *nef* is expressed as an early gene product in the HIV-1 lifecycle. Nef is a 27 kilodalton adaptor protein that has been implicated in altering the intracellular trafficking pathways of many immune molecules, such as class I and II major

histocompatibility complex proteins (MHC-I and MHC-II), CD4, CD28, and DC-SIGN (23-27). It has been shown that specific sequences in the cytoplasmic tails of each molecule are important for responsiveness to Nef (28,29). Nef achieves conditions optimal for viral replication and spread by modulating the surface expression of MHC-I and CD4 (discussed below).

HIV-1 Immune Evasion

The HIV-1 protein Nef is indispensable for viral fitness and persistence (30,31). Expression of Nef prematurely activates and matures CD4⁺ T lymphocytes therefore causing non-specific immune activation and dysfunction(32,33). Non-human primate research has revealed that the simian immunodeficiency virus (SIV) Nef protein is an important factor required for eventual immune collapse. In fact, some rhesus macaques infected with a Nef-deleted (Δnef) strain of SIV do not progress to AIDS (34).

In addition there is a cohort of blood transfusion recipients who was exposed to an HIV-1 variant (35) that contained a significant deletion in the viral genome including part of the *nef* gene and the long terminal repeat (LTR) (36). Decades after infection and without anti-retroviral treatment (37), none of these patients have progressed to AIDS, but some do have reduced CD4⁺ T cell counts (38,39). These patients are considered long term non-progressors (LTNPs). The combination of non-human primate research and longitudinal patient cohort studies has revealed the importance of Nef in the progression from HIV disease to AIDS.

In healthy individuals, specialized CD8⁺ cells in the immune system, cytotoxic T lymphocytes (CTLs), function by distinguishing “self” from “non-self” peptide signals found on the surface of cells. Each of these signals is presented on the cell surface by a class of molecules called major histocompatibility complexes-class I (MHC-I). In a healthy cell, MHC-I presents cellular peptides that are normally made in the cell (“self” peptides) and typically do not activate a CTL response. In a virally infected cell, MHC-I presents peptides produced by the virus (“non-self” peptides). Once the combination of the T cell receptor (TCR) and CD8 on a CTL recognize a “non-self” signal presented by

MHC-I, the CTL kills the virally infected cell and prevents further spread of the virus (reviewed in (40)).

Many viruses that infect humans have evolved ways to evade CTL recognition by their hosts. For example, the adenovirus E3 gene causes endoplasmic reticulum (ER) retention of MHC-I molecules through a retention signal in the E3 protein (41). A herpes simplex virus protein IPC47 inhibits the transporter associated with antigen processing (TAP) (42), thus causing ER retention of MHC-I lacking antigenic peptide (43). The Kaposi's sarcoma-associated virus (HHV-8) proteins MIR-1 and MIR-2 ubiquitinate lysines in the cytoplasmic tail of MHC-I, thus causing rapid endocytosis and degradation of MHC-I (reviewed in (44)).

HIV-1 requires the expression of Nef *in vivo* for maintenance of viral survival and spread but Nef is not needed for the production of infectious virus *in vitro* (45), thus Nef may function to counteract the host immune response. Two groups in 1989 observed HIV-1 downmodulation of MHC-I molecules (46,47). In 1996, Schwartz *et al* discovered that the Nef protein was responsible for reducing MHC-I surface expression by deleting the *nef* gene from the HIV-1 genome (24). In a seminal research article, Collins *et al* revealed that in HIV-1-infected peripheral blood mononuclear cells (PBMCs) Nef activity rescues infected cells from anti-HIV CTL lysis (48). Cells that were infected with an HIV-1 variant containing a frameshift mutation in *nef* were efficiently lysed by CTLs. This work proved that Nef downmodulates MHC-I molecules specifically to allow HIV-1 infected cells to evade lysis by CTLs (48).

In contrast to CTL antigen recognition, natural killer (NK) cells monitor the levels of MHC-I on the cell surface and lyse target cells that have low levels of MHC-I molecules. Low surface expression of the MHC-I allotypes HLA-A and HLA-B is important for NK cell activation, whereas HLA-C and HLA-E are NK cell inhibitory molecules (reviewed in (49)). Nef has been shown to directly interact with an amino acid sequence in the cytoplasmic tail of MHC-I molecules bearing a Y₃₂₀SQAASS₃₂₆ sequence (29). HLA-A and HLA-B molecules contain this amino acid sequence in their cytoplasmic tails and therefore are bound and downmodulated by Nef (28,29,50). HLA-C and HLA-E, which do not have this required amino acid sequence (28,29), selectively remain on the plasma membrane to inhibit NK cell recognition (50). Thus, Nef is able to

specifically inhibit CTL and NK cell lysis by selectively downmodulating certain MHC-I molecules.

Domains Required for Functional Nef

Summarized in Figure 1.3, multiple domains of Nef have been implicated in MHC-I and CD4 downmodulation. Nef can be divided into the anchor domain, the core domain, and the C-terminal flexible loop (51). Two sites in Nef are required for all of Nef's functions. First, Nef is myristoylated at amino acid position 2, allowing for membrane association (52). Through microscopic analysis, it was shown that Nef associates with membranes and co-localizes with MHC-I in a perinuclear region (28,29,53,54). Next, an oligomerization domain (D₁₂₃) is required to form homodimers of Nef (55). If either of these sites is mutated (G₂A or D₁₂₃G), Nef is no longer functional. Three regions of Nef, an N-terminal α -helix (R₁₇ERM₂₀RRAEPA₂₆ and specifically M₂₀) (56,57), an acidic cluster (E₆₂₋₆₅)(58), and a polyproline repeat (P_{72/75/78}) (57), are required for Nef's ability to downmodulate and bind to the cytoplasmic tail of MHC-I (reviewed in (59), (60)) (discussed below). All of the domains required for MHC-I downmodulation are either in the N-terminal anchor domain or the core domain. A discrete binding site on Nef needed to interact with MHC-I has not yet been determined. Thus, this interaction may require a specific structure that is stabilized by a combination of various Nef domains.

Mutagenesis of domains in Nef has revealed that there are genetically separable mechanisms that affect MHC-I and CD4 trafficking as shown in Figure 1.3. A hydrophobic pocket (including WL_{57,58}) in Nef has been shown by NMR analysis to bind the cytoplasmic tail of CD4 (61,62). Nef's dileucine (E₁₆₁xxxLL₁₆₆) and diacidic motif (DD_{175,176}) are required for CD4 downmodulation (discussed below), but are dispensable for MHC-I downmodulation. A second diacidic motif (EE_{155,156}) in Nef has been implicated in CD4 trafficking to acidic compartments for degradation (reviewed in (59), (63)).

Nef Disrupts Cellular Factors Important for Intracellular Trafficking

Nef disturbs a host of cellular processes by directly interacting with host cell proteins and commandeering cellular trafficking pathways reviewed in Figure 1.4. Intracellular trafficking can be broken down into clathrin-dependent and clathrin-independent vesicle formation and transport. Clathrin-coated vesicles can transport cargo from the *trans*-Golgi network, plasma membrane, or endosomal network. Clathrin-associated adaptor proteins (APs) are composed of four subunits: two heavy subunits ($\beta 1$ or $\beta 2$ and AP-1 γ , AP-2 α , or AP-3 δ), one medium subunit (μ), and one small subunit (σ) (64-66). The four subunits combine to function as a heterotetrameric adaptor protein complex that recognizes Yxx ϕ (Y, tyrosine; ϕ , bulky hydrophobic amino acid; x, any amino acid) and [D/E]xxxLL (D, aspartic acid; E, glutamic acid; L, leucine) sorting signals and recruits clathrin coats. AP-1 cycles proteins between the *trans*-Golgi network and endosomes (67-69). AP-2 localizes to the plasma membrane and internalizes sorting signals into endosomes (65). AP-3 localizes to endosomes and is thought to transport proteins into acidic, degradative compartments (70).

Multiple domains in each adaptor protein complex are important for proper function. The crystal structures of AP-1 (71) and AP-2 (72) have been solved, revealing physically separate sites for sorting signal recognition on adaptor proteins: μ contains a tyrosine and hydrophobic binding pocket (TBP) which recognizes Yxx ϕ signals (73,74); the β subunit (75,76) or either γ/σ_1 or α/σ_2 hemi-complexes (77,78) bind dileucine motifs [D/E]xxxLL.

The regulation of each adaptor protein is cyclical depending on the phosphorylation state of their respective μ and β subunits of each adaptor protein (79,80). Phosphorylation of the μ subunit changes the adaptor protein state into a relaxed conformation, thus revealing the TBP and increasing its affinity for tyrosine based sorting signals (71,80-82). Specifically, mutating the phosphorylation site on AP-2 $\mu 2$ (T₁₅₆) reduces endocytosis of Yxx ϕ trafficking signals (83,84). In contrast, the β subunit is maintained in an unphosphorylated state during clathrin coated vesicle formation. Upon vesicle release from the compartment of origin the β subunit is phosphorylated (80) and the adaptor proteins are released into the cytoplasm in a step called uncoating (85).

The two heavy chains $\beta 1$ or $\beta 2$ and $\gamma 1$, $\alpha 2$, or $\delta 3$ are bound by clathrin through a clathrin box motif (86,87). The N-terminus of each of the heavy chains $\gamma 1$, $\alpha 2$, or $\delta 3$ also interacts with phosphoinositides found on membranes in the appropriate cellular localization for each adaptor protein (reviewed in (88)). For example, the *trans*-Golgi membranes are enriched in phosphoinositol-4-phosphate which has been shown to be required for AP-1 recruitment (89). Likewise, the plasma membrane is enriched with phosphoinositol-4,5-bisphosphate, which is required for AP-2 localization (90).

Initially yeast two-hybrid assays revealed that Nef's dileucine motif interacts with the μ subunit of AP-1 and AP-3 (28,78,91-97). Additionally, yeast three-hybrid analysis revealed that Nef's dileucine motif interacts with the $\gamma/\sigma 1$ or $\delta/\sigma 3$ hemicomplexes (78). Shown through microscopic analysis, a Nef-GFP fusion protein co-localizes well with AP-1 γ and AP-3 δ , and weakly with AP-2 α (91) and a CD8-Nef fusion protein stabilizes AP-1 γ and AP-3 δ on membranes in a Nef dileucine motif-dependent manner (96).

In addition to the clathrin-associated adaptor proteins, the small GTPases, ADP-ribosylation factors (ARFs), are important for cellular control of assembly and disassembly of various intracellular trafficking complexes (98,99). ARFs are important for both clathrin-coated (100-103) and non-clathrin-coated (104,105) vesicle formation. ARF activation and recruitment to cellular membranes is cyclical and regulated by its GTP binding state. Guanine nucleotide exchange factors (GEFs) mediate the recruitment of GTP to ARF and are required for overall Golgi structure ((106,107) and reviewed in (108)). Binding GTP causes ARF to undergo a conformational change exposing a myristoyl group that inserts into membranes. GTPase-activation proteins (GAPs) are important to aid ARFs in GTP hydrolysis, thus inactivating ARFs (109). Dominant inhibitory mutants of ARFs have been shown to be locked in either a GDP-bound, inactive state or a GTP-bound, active state (110,111).

ARF-1 is a clathrin regulatory protein that upon activation inserts into membranes and subsequently recruits AP-1 or coat protein complex-I (COP-I) coatomers (112). Pan ARF-1 inactivation during mitosis causes Golgi dissociation and appropriate cellular localization of proteins involved in cytokinesis (113). As mentioned previously, Nef stabilizes AP-1 on membranes in a Nef dileucine motif-dependent manner. Additionally, the GEF inhibitor brefeldin A (114) is unable to inhibit AP-1 recruitment to the dileucine

motif in Nef (96), revealing that ARF-1 is not involved. In the case of MHC-I downmodulation, Nef's dileucine motif is dispensable, thus downmodulation of MHC-I by Nef could be ARF-1 dependent.

ARF-6 has been implicated in MHC-I downmodulation by Nef (discussed below) (115). ARF-6 localizes to the plasma membrane and is involved in clathrin-independent endocytosis and recycling (116). Activation of ARF-6 has been implicated in the formation of cellular protrusions and ruffling (117,118). In addition, ARF-6 regulates the cellular localization of phosphoinositol-4,5-bisphosphate, which plays a key role in Gag localization to the plasma membrane during assembly and budding of HIV (119).

Another instance of clathrin-independent vesicle formation seen in Figure 1.4, COP-I and COP-II coatomers traffic cargo from either the Golgi or ER, respectively (120,121). In a less traditional mechanism, COP-I coatomers localize to low pH endosomes in an ARF-1-dependent manner (122,123). Shown in Figure 1.4, COP-I coatomers are implicated in recycling endosome function (124) and transport from the endolysosomal network into multivesicular bodies (125). The COP-I subunit β -COP was first identified as a binding partner for Nef in a yeast two-hybrid screen (126). Although conflicting studies have disputed the role of a diacidic motif (EE_{155, 156}) in Nef for β -COP recruitment (127,128), previous research links functional ARF-1 and this diacidic motif in Nef to CD4 localization to degradative compartments (63,129).

Through a yeast two-hybrid screen, Nef's other diacidic motif (DD_{175,176}) was shown to directly interact with the H subunit of the universal proton pump (V1H) (130-132). V1H is a regulatory protein that has been shown to be important for degradation of endocytosed cargo by binding to and acidifying endosomes and lysosomes (133). Additionally, V1H was shown to mediate an interaction between Nef's C-terminal flexible loop and AP-2 μ 2. Deletion of the C-terminal flexible loop of Nef abolished the three-way complex formation, and fusion of V1H in its place recovered AP-2 μ 2 recruitment (132). In a more straightforward scenario, yeast three-hybrid assays have revealed that the diacidic motif (DD_{175,176}) directly interacts with AP-2, thus simplifying the mechanism by which Nef recruits AP-2 (128). Links between Nef and β -COP and V1H suggest that Nef commandeers degradative machinery in HIV-1 infected cells.

Discussed further below, Nef has been shown to interact with PACS-1 and PACS-2 through its acidic cluster E₆₂₋₆₅ (54). The phosphofurin acidic cluster sorting (PACS) proteins 1 and 2 were originally discovered by studying proteins that bound to the cytoplasmic tail of furin (134). The natural role of PACS-1 and PACS-2 is to bind to acidic clusters in cargo proteins, recruit AP-1 or AP-3 (135), and localize them to the *trans*-Golgi network (136). While it has yet to be found in coated vesicles (cited as data not shown in (137)), PACS has been shown to recruit AP-1 to a protein important for vesicle-membrane fusion, the SNARE vesicle-associated membrane protein (VAMP)-4 (138).

Nef Enhances Endocytosis Rates of CD4

Downmodulation of the viral co-receptor CD4 is indispensable for HIV Env incorporation into virions and release of infectious virus (13,14). Depicted in Figure 1.5, CD4 transports through the secretory pathway normally (32,139) and Nef rapidly internalizes CD4 into endosomal compartments (140). Importantly, domains in Nef that are required for CD4 downmodulation are also important for HIV replication and pathogenesis (141,142). The hydrophobic pocket (WL_{57,58}) in Nef directly interacts with the QIRKLLSEKKT region of the cytoplasmic tail of CD4 (62,143-146). CD4 accumulates in AP-2 positive endocytic endosomes in HIV infected cells (53). In addition, RNAi knockdown of AP-2 (147-149) or clathrin (149) or expression of dominant negative Eps15 and Dynamin endocytosis inhibitors (150), hinder Nef-induced downmodulation of CD4. However, a three-way complex between CD4, Nef, and AP-2 has not yet been isolated. Thus, the mechanism by which Nef functions on CD4 remains correlative between interaction and downmodulation assays. Nef directly interacts with purified AP-2 (149) and yeast three-hybrid direct interaction assays have mapped the regions of AP-2 (151) and Nef that interact to include the original dileucine motif as well as a C-terminal diacidic motif (DD_{175,176}) (128). When the C-terminal flexible loop in Nef is deleted, Nef cannot downmodulate CD4. In contrast, when the C-terminal flexible loop in Nef is replaced by V1H, Nef's function is rescued and can downmodulate CD4 (130,132) presumably by replacing Nef's AP-2 recruitment domain. Additionally, Nef's

diacidic motif (EE_{154,155}) recruits β -COP (63,129) to degrade CD4 in lysosomal compartments (152). However, subsequent work has argued through yeast two-hybrid analysis that Nef's diacidic domain (EE_{154,155}) is not required to interact with β -COP (127).

Nef Downmodulation of MHC-I: Evidence for an Endocytic Mechanism

Since Nef was first implicated in the reduction of MHC-I surface expression, an abundance of research has been performed and two opposing models have emerged that aim to explain the mechanism by which Nef functions. The first model of downmodulation (Figure 1.6) involves an increase in the rate of MHC-I internalization from the plasma membrane. The second model of downmodulation (Figure 1.7) entails an obstruction of normal MHC-I secretion to the plasma membrane. The first reports of MHC-I downmodulation by Nef revealed that Nef does not alter the rate of MHC-I synthesis or trafficking through the ER or *cis*-Golgi, but does decrease MHC-I stability over time through lysosomal degradation (cited as data not shown (24)). Furthermore, Nef causes an accumulation of MHC-I in perinuclear and endosomal compartments and enhances the rate of endocytosis in HIV infected cells (24).

Because CD4 and MHC-I progress through the secretory pathway and accumulate in similar cellular compartments (139,140,153), it was hypothesized that Nef utilizes a similar pathway to affect both molecules (58). However as summarized in Figure 1.3, Greenberg *et al* determined that domains in Nef needed for CD4 and MHC-I downmodulation are separable and that accumulated MHC-I co-localizes with AP-1, rather than AP-2 (58). The acidic cluster (E₆₂₋₆₅), the polyproline SH3 binding domain (P_{72/75/78}) (58,60), and the N-terminal α -helix (R₁₇ERM₂₀RRAEPA₂₆ specifically M₂₀) (57,60) in Nef were all shown to be important domains for MHC-I downmodulation. In T lymphocytes and macrophages, MHC-I is spontaneously endocytosed and recycled back to the plasma membrane at high rates in an AP-2 dependent manner (154,155). However, treatment with a dominant negative dynamin (156) or an AP-2 TBP dominant negative mutant molecule (115) revealed that Nef-induced MHC-I endocytosis is clathrin independent. Thus Nef affects MHC-I and CD4 through different mechanisms.

In uninfected HeLa cells, MHC-I co-localizes with ARF-6 at the plasma membrane (116). Pan-activation of ARF-6 in HeLa cells through AlF₄ treatment, constitutively active ARF-6 (Q₆₇L) or by dominant negative ARF-6 GEF ARNO (E₁₅₆K) overexpression endocytosis of MHC-I by Nef is inhibited (115). In the astrocytic A7 cell line, it was also shown that PI-3-Kinase inhibitors are able to impair GTP loading on to ARF-6 and subsequent MHC-I internalization (115). In agreement with previously mentioned functional analysis (58), the acidic cluster (E₆₂₋₆₅) and polyproline (P_{72/75/78}) Nef mutants were shown to be unable to internalize MHC-I (115). Interestingly, the Nef mutant M₂₀A, which was incapable of downmodulating steady state levels of MHC-I (56,57), was able to endocytose MHC-I. Subsequent steps of sorting to AP-1 containing compartments were impaired with this mutant (115), thus suggesting PI-3-Kinase and ARF-6 are required for endocytosis into endosomes and M₂₀ is required for AP-1 recruitment allowing for endosome to *trans*-Golgi localization (115,157).

The acidic cluster (E₆₂₋₆₅) and polyproline (P_{72/75/78}) domain in Nef were required for sequential steps leading to enhanced activation of ARF-6 (157). Through purified protein interaction analysis, Nef's acidic cluster E₆₂₋₆₅ bound to PACS-1 and PACS-2. RNAi directed toward either PACS-1 or PACS-2 reduced the starting surface expression of HLA-A2 in CD4⁺ primary T cells and inhibited any further downmodulation by Nef (158). In the absence of PACS-1 or PACS-2 in either RNAi treated HeLa cells or in PACS-2^{-/-} mouse splenocytes, Nef was able to endocytose MHC-I but was unable to transport it to AP-1 containing compartments. This suggests that PACS-1 and PACS-2 are not responsible for the endocytosis step, but are required for endosomal retrograde transport to the *trans*-Golgi network.

The polyproline domain (P_{72/75/78}) in Nef has been shown to recruit the *trans*-Golgi localized Src family kinase, Hck (159). Mutating the polyproline domain completely abolishes MHC-I internalization and the Nef-Hck interaction (157,159). In CD4⁺ H9 cells, it has been shown that the Nef-Hck complex recruits and activates the tyrosine kinase ZAP-70, which then activates PI-3-Kinase causing MHC-I internalization (157). In Jurkat CD4⁺ T cells, Nef was shown to be in complex with PI-3-Kinase and ZAP-70. Inhibiting any of these proteins through chemical or RNAi treatment in H9 and

CD4⁺ primary T cells reduced the amount of MHC-I downmodulation caused by Nef (157).

The overall model for MHC-I endocytosis by Nef is shown in Figure 1.6. Confounding this complex model, conflicting results have reported that overexpression of ARF-6 dominant negative mutants does not specifically affect Nef-induced downmodulation of MHC-I (160). It was determined that ARF-6 Q₆₇L non-specifically activates downstream effectors causing an overall inhibition of membrane trafficking in cells (160). Additionally, recent knock down data disputes PACS-1 and PACS-2 involvement in sorting proteins containing acidic clusters and Nef-induced downmodulation of MHC-I (161). Furthermore, comprehensive analyses in physiologically relevant cell systems indicate that endocytosis plays a lesser role in downmodulating MHC-I from the plasma membrane (discussed below) (162).

Evidence That Nef Redirects MHC-I into the Endolysosomal Network

The relative amount of downmodulation in HeLa cells (2-4 fold reduction (115)) is not comparable to the fold change in MHC-I surface expression needed to allow HIV infected cells to escape CTL lysis (up to 300-fold reduction (cited as data not shown (48))). Previous reports in Nef-expressing cells of MHC-I localizing to the *trans*-Golgi and AP-1-containing vesicles suggested that Nef could be causing *trans*-Golgi network retention.

A Nef-induced block in transport to the plasma membrane was first shown in 373MG astrocytic cells through microscopic analysis (163). First, MHC-I was accumulated in the *trans*-Golgi by incubating at 20°C causing a temperature sensitive block in transport to the plasma membrane. Subsequently, cells were incubated at 37°C for varying time points, fixed, permeabilized, and stained for MHC-I to reveal the cellular localization and speed of transport to the plasma membrane. In the absence of Nef, MHC-I relocated to the plasma membrane within 15 minutes of the permissive temperature shift. MHC-I in Nef-transduced cells was maintained in the characteristic perinuclear compartment for as long as the assay measured, 60 minutes (163).

A comprehensive study was performed comparing Nef's effects on MHC-I endocytosis, recycling, and transport in various cell lines (162). The results revealed a striking difference in Nef's mechanism of action in HeLa cells versus the CEM-SS T cell line, a CD4⁺ T lymphoblastoid cell line. Unlike in HeLa cells, Nef was unable to increase the rate of endocytosis or inhibit recycling of MHC-I in CEM-SS T cells (162). By analyzing radioactive pulse-labeled, biotinylated surface proteins in CEM-SS T cells, it was discovered that Nef caused an 8-fold reduction in MHC-I transport to the plasma membrane (162). These data were further confirmed in primary T lymphocytes through a flow cytometric-based transport assay (162), thus revealing verification of this mechanism in a physiologically relevant cell type.

The HLA-A2 cytoplasmic tail is phosphorylated at specific serines *in vivo* upon reaching the plasma membrane (164). Nef preferentially binds hypophosphorylated forms of HLA-A2 and inhibits these phosphorylation events (165). By binding MHC-I early in the secretory pathway (165), Nef induces MHC-I accumulation in AP-1-containing compartments (58), thus blocking MHC-I transport from the *trans*-Golgi to the plasma membrane (162,163). In addition, RNAi directed toward AP-1 μ 1 reversed HLA-A2 downmodulation in both astrocytic and CEM-SS T cells (153). In fact, Nef was shown to recruit AP-1 to the cytoplasmic tail of HLA-A2 in primary T cells (153). Illuminating experiments revealed that normal secretory trafficking of MHC-I through HeLa cells was too fast for Nef to recruit AP-1 to the MHC-I cytoplasmic tail. A temperature shift to 26°C slowed the secretory pathway enough that Nef could recruit AP-1 to MHC-I (165), thus revealing HeLa cells downmodulate MHC-I through a different mechanism.

Yeast two-hybrid interaction assays and cellular localization through microscopic analysis demonstrated Nef binding to AP-1 and AP-3 through Nef's dileucine motif (78,91-97). In contrast, MHC-I downmodulation and AP-1 recruitment in CEM-SS cells was independent of Nef's dileucine motif (58,60,153), suggesting a different domain was required for AP-1 recruitment. Furthermore, the tyrosine (Y₃₂₀SQA) in HLA-A2 was absolutely required for AP-1 recruitment (153) despite the absence of a canonical Yxx ϕ sorting signal. Thus, a cryptic Yxx ϕ sorting signal in the cytoplasmic tail of HLA-A and HLA-B allotypes is responsible for responsiveness to Nef (28).

Each domain in Nef functionally required for MHC-I downmodulation was also required for Nef to interact with MHC-I (60); therefore, a *cis* expression system (HLA-A2 fused to Nef) was employed to explore domains in Nef that were required for AP-1 recruitment. Similar to the *trans*-expression system, Nef's dileucine motif was shown to be dispensable for AP-1 recruitment. Interestingly, the *cis* expression system demonstrated that both the acidic cluster (E₆₂₋₆₅) and the polyproline repeat (P_{72/75/78}) were also dispensable for AP-1 recruitment. Finally, it was shown that the N-terminal α -helix and specifically M₂₀, were absolutely required for AP-1 recruitment. Therefore, two domains are responsible for AP-1 recruitment and subsequent downmodulation of MHC-I: Y₃₂₀SQA in the cytoplasmic tail of HLA-A2 and M₂₀ in Nef (153).

As compiled in Figure 1.7 (153), Nef recruits AP-1 to the cytoplasmic tail of MHC-I in the *trans*-Golgi network and targets MHC-I containing vesicles into the endosomal network controlled by AP-1. In agreement with previous data (24), Nef induces degradation within 4 hours of MHC-I synthesis and it is reversed with lysosomal inhibitors (153). This suggests that AP-1 is involved in the re-routing of MHC-I into the endosomal network, however accelerated degradation of MHC-I remains to be explained (24,153),

Conclusion

Nef has been shown to be important for progression from HIV to AIDS because it is important for efficient viral fitness and spread. Nef downmodulates MHC-I to allow HIV infected cells to evade detection by the immune system. Two models exist that aim to explain Nef's mechanism of action on MHC-I: enhancing MHC-I endocytosis rates or re-directing MHC-I transport from the plasma membrane to the endolysosomal network. Further research needs to be explored to confirm or disprove either of the two mechanisms of downmodulation. Understanding the mechanism by which Nef evades immune system recognition is important for drug discovery that could allow for clearance of HIV in infected individuals.

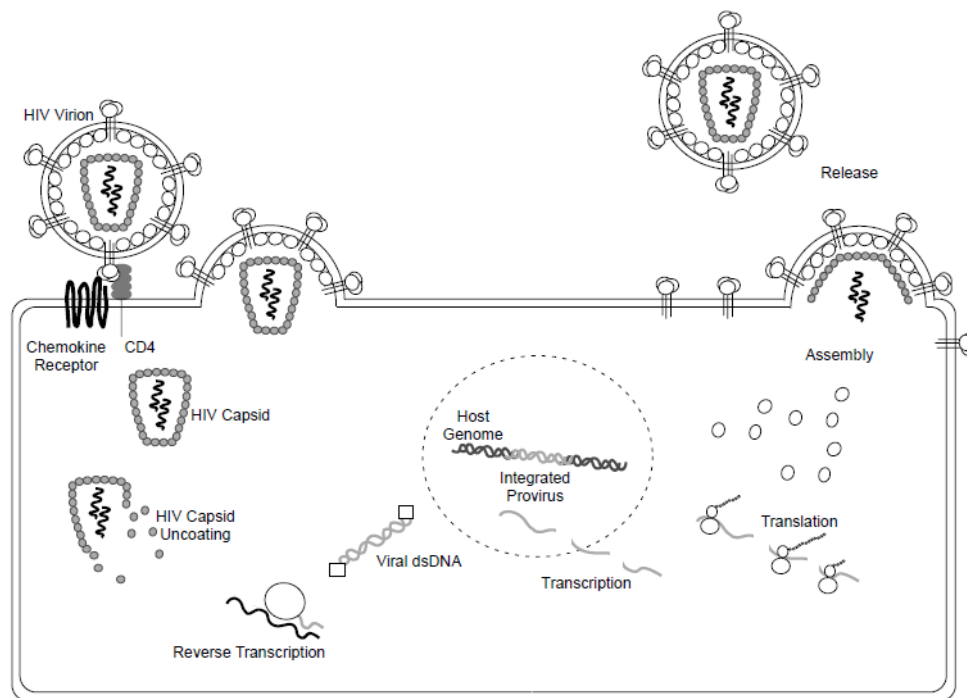


Figure 1.1. Schematic representation of the HIV-1 lifecycle.

Major steps in HIV replication are indicated in a highly simplified form. The explanation of each step is described in the text.

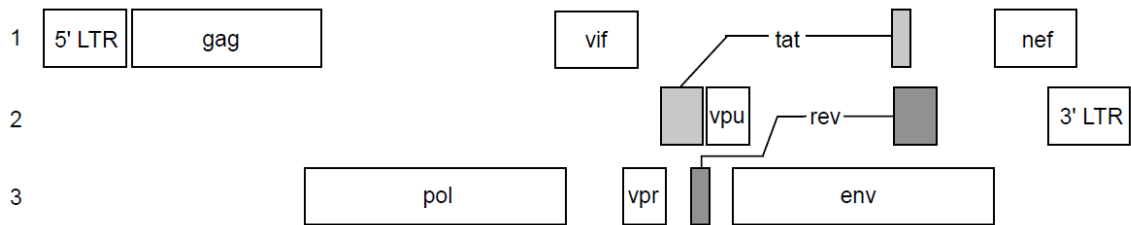


Figure 1.2: The genome of HIV-1.

Three reading frames are shown to reveal HIV-1 genes and their relative genome locations. Open reading frames are shown as rectangular boxes. The spliced reading frames, tat and rev, are shown as gray boxes.

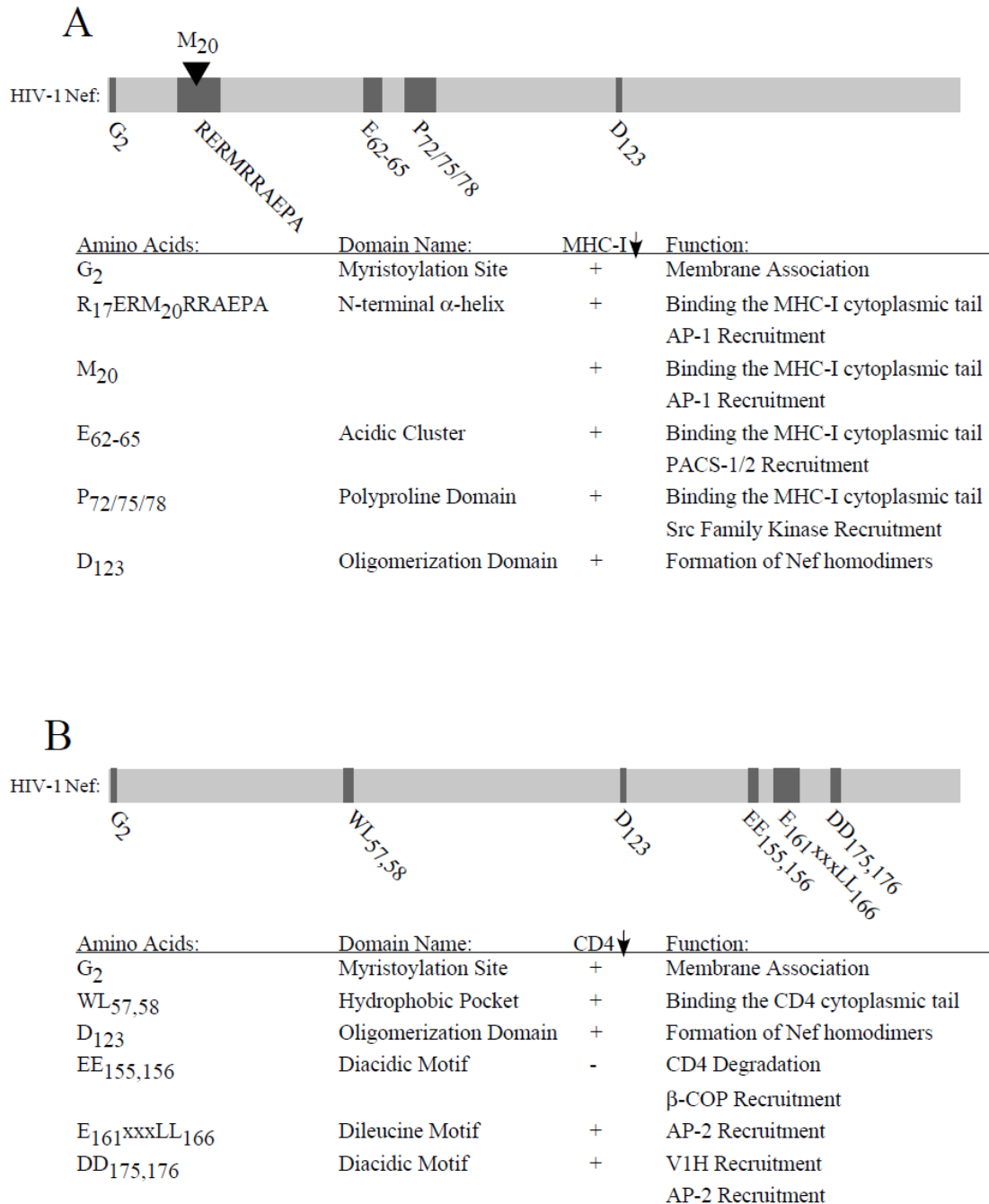


Figure 1.3. Identity and summary of the functions associated with each domain of Nef.

(A) Nef domains involved in affecting MHC-I. + or -, denotes whether each domain is required for reducing the surface expression of MHC-I. (B) Nef domains involved in affecting CD4. + or -, denotes whether each domain is required for reducing the surface expression of CD4.

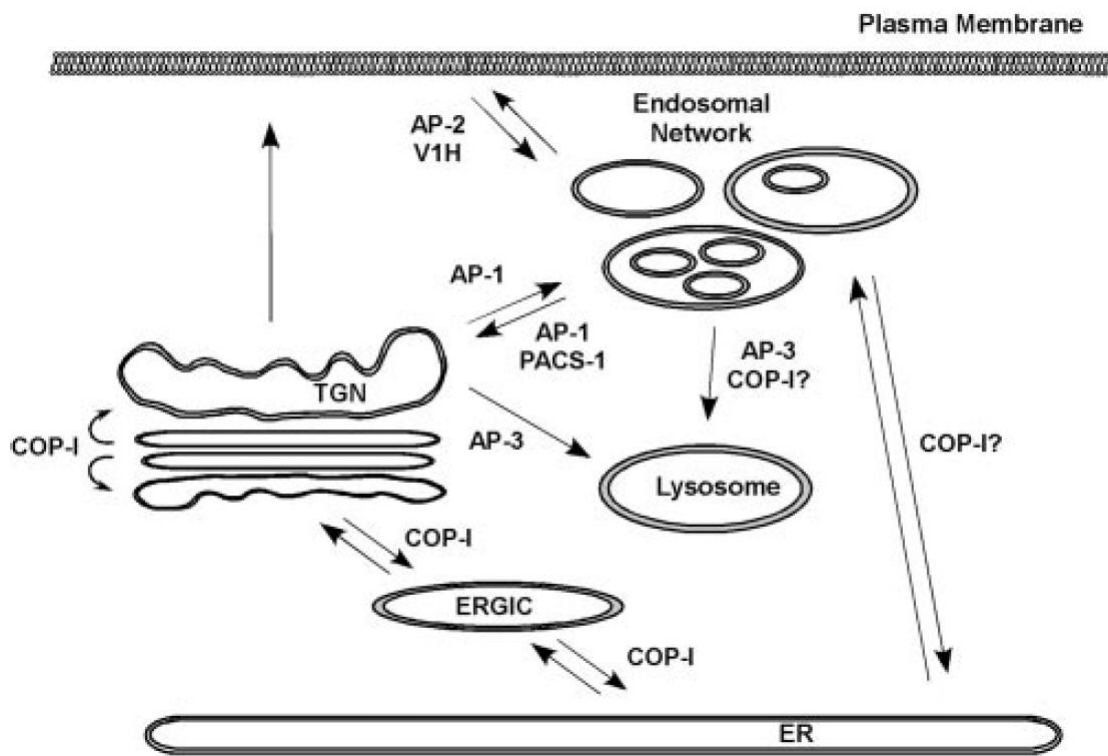


Figure 1.4. Intracellular trafficking pathways. Reprinted with permission from reference (166).

A simplified representation of factors involved in transporting cargo between intracellular compartments.

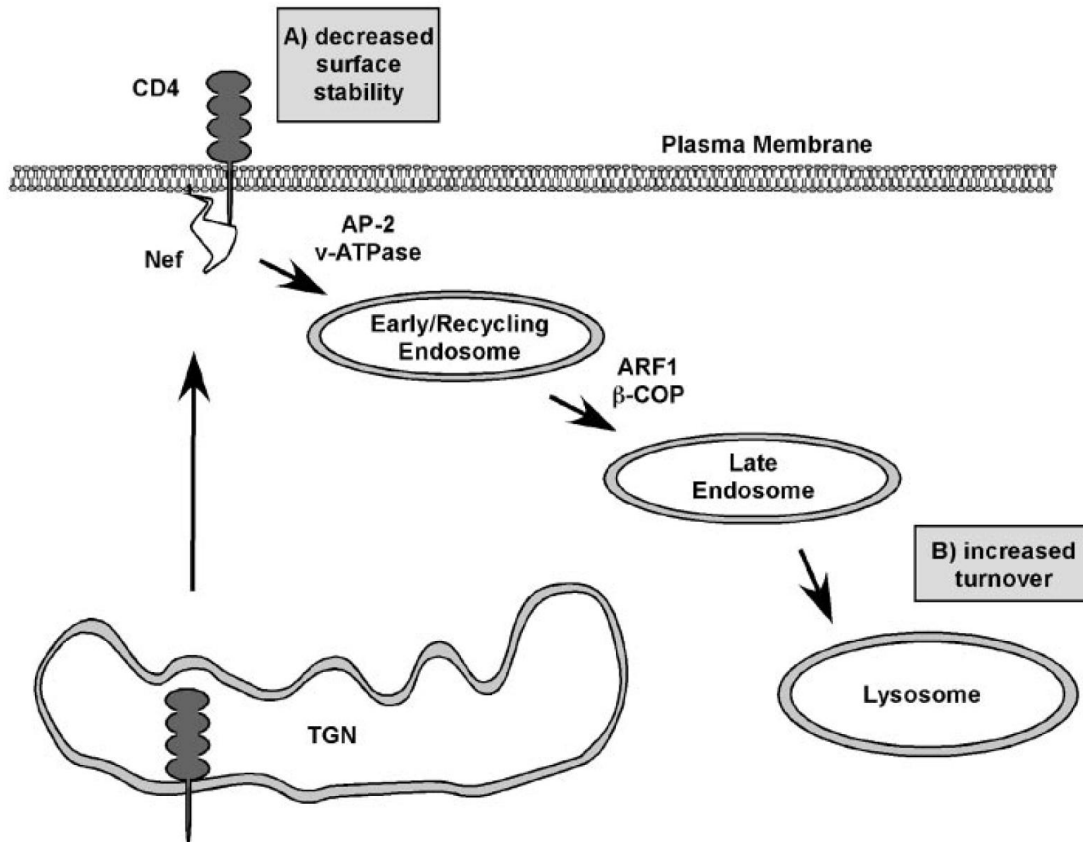


Figure 1.5. Nef enhances CD4 internalization. Reprinted with permission from reference (166).

(A) Nef binds to the cytoplasmic tail of CD4 and enhances its endocytosis rate by recruiting the clathrin associated adaptor protein AP-2. (B) Nef recruits β -COP and ARF-1 to localize CD4 to acidic compartments and allow for subsequent degradation.

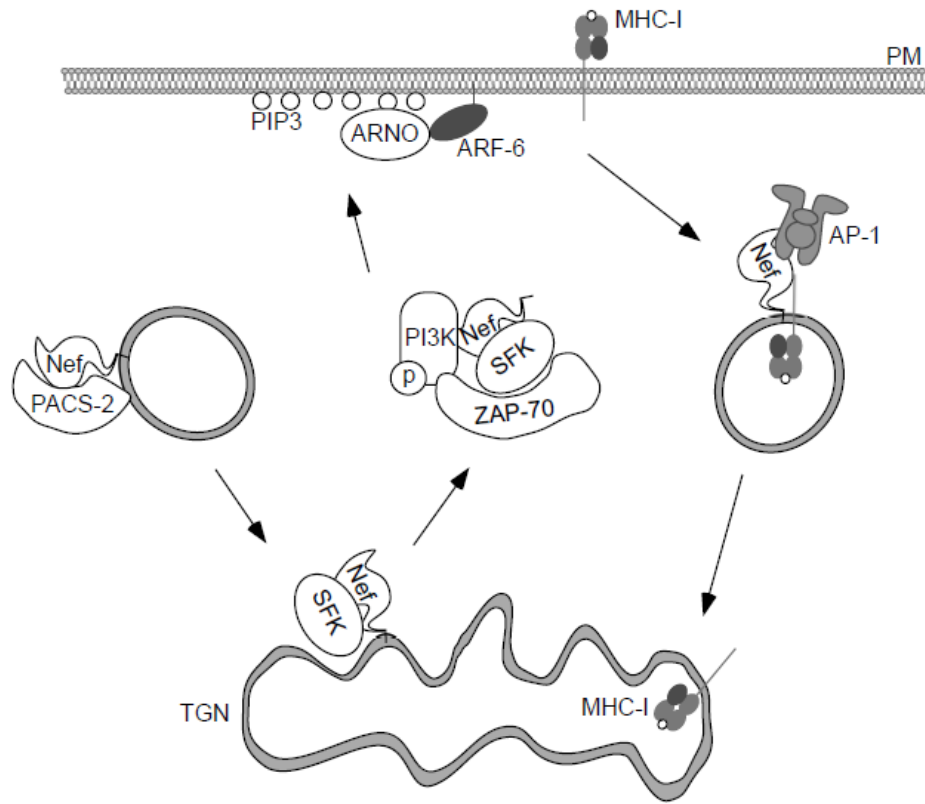


Figure 1.6. Nef-induced endocytosis of MHC-I.

Nef is bound by the acidic cluster sorting protein, PACS-2, and localized to the *trans*-Golgi network (TGN). Nef binds to the Src Family kinase (SFK), Hck, which activates the tyrosine kinase ZAP-70. ZAP-70 then binds to and activates PI-3-Kinase. PI-3-Kinase creates PIP₃ on the inner leaflet of the plasma membrane (PM) which recruits the ARF-6 GEF, ARNO, subsequently recruiting and activating ARF-6. MHC-I is then endocytosed by ARF-6 into endosomes. Nef then recruits AP-1 which transports MHC-I to the TGN.

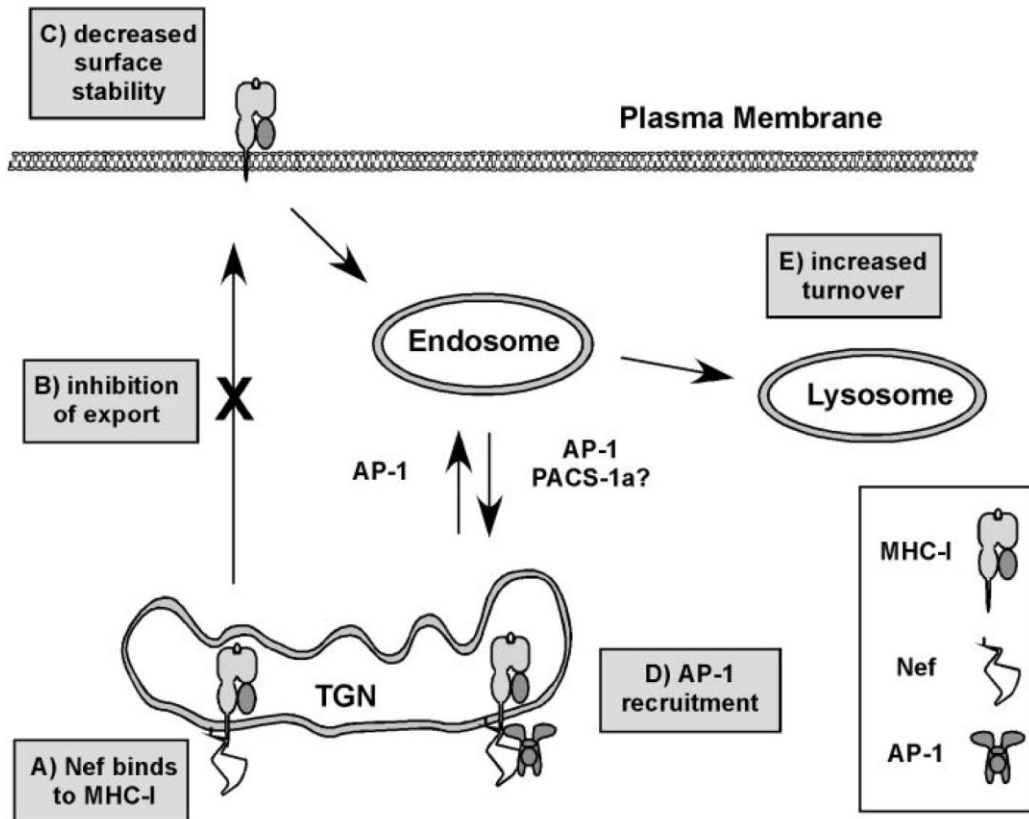


Figure 1.7. Nef re-routes MHC-I into the endolysosomal network. Re-printed with permission from reference (166).

(A) Nef binds to the cytoplasmic tail of MHC-I early in the secretory pathway. (B) Nef blocks normal secretion of MHC-I to the plasma membrane. (C) Nef decreases the surface stability of MHC-I. (D) Nef recruits AP-1 to redirect MHC-I into the endolysosomal network.

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CHAPTER II

The Tyrosine-Binding Pocket in the AP-1 μ 1 Subunit Is Necessary For Nef to Recruit AP-1 To the MHC-I Cytoplasmic Tail

Abstract

To evade the anti-HIV immune response, the HIV Nef protein disrupts MHC-I trafficking by recruiting the clathrin adaptor protein 1 (AP-1) to the MHC-I cytoplasmic tail. Under normal conditions, AP-1 binds dileucine and tyrosine signals (YXX ϕ motifs) via physically separate bindings sites. In the case of the Nef-MHC-I complex, a tyrosine in the HLA-A2 cytoplasmic tail (Y₃₂₀SQA) and a methionine in Nef (M₂₀) are absolutely required for AP-1 binding. Also present in Nef is a dileucine motif, which does not normally affect MHC-I trafficking and is not needed to recruit AP-1 to the Nef-MHC-I-complex. However, evidence is presented here that this dileucine motif can be activated by fusing Nef to the HLA-A2 tail in *cis*. Thus, the inability of this motif to function in *trans* likely results from a structural change that occurs when Nef binds to the MHC-I cytoplasmic tail. The physiologically relevant tyrosine-dependent recruitment of AP-1 to MHC-I, which occurs whether Nef is present in *cis* or *trans*, was stabilized by the acidic and polyproline domains within Nef. Additionally, amino acids A₃₂₄, and D₃₂₇ in the cytoplasmic tails of HLA-A and B (but not HLA-C and HLA-E) molecules also stabilized AP-1 binding. Finally, mutation of the tyrosine-binding pocket in the μ subunit of AP-1 created a dominant negative inhibitor of Nef-induced down-modulation of HLA-A2 that disrupted binding of wild type AP-1 to the Nef-MHC-I complex. Thus, these data provide evidence that Nef binding to the MHC-I cytoplasmic tail stabilizes the interaction of a tyrosine in the MHC-I cytoplasmic tail with the natural tyrosine-binding pocket in AP-1.

Introduction

Cytotoxic T-lymphocytes (CTLs) recognize and lyse virally infected cells by detecting viral peptides presented at the cell surface in association with host major histocompatibility complex class I proteins (MHC-I). The HIV-1 Nef protein reduces cell surface expression of MHC-I (1-3) and thus limits the ability of anti-HIV CTLs to recognize and lyse HIV-infected primary T cells (4). Nef disrupts the trafficking of MHC-I HLA-A and HLA-B molecules, but allows the normal expression of HLA-C and HLA-E. It has been postulated that continued expression of HLA-C and HLA-E may limit recognition by natural killer cells (5,6). The differential effects of Nef on MHC-I molecules results from variations in MHC-I cytoplasmic tail sequences. There are three key amino acids in the cytoplasmic tails of Nef-responsive MHC-I molecules (Y₃₂₀, A₃₂₄ and D₃₂₇). A subset of these amino acids is missing in HLA-C and HLA-E, causing them to be resistant to Nef (5,6).

It has not yet been possible to define a discrete binding site on Nef that interacts with MHC-I. Thus far, mutation of each domain in Nef that has been implicated in MHC-I down-modulation (M₂₀A, V₁₀EΔ17-26, D₁₂₃G, E₆₂₋₆₅Q, P_{72/75}A and P_{75/78}A) results in disruption of Nef binding to MHC-I (7). Thus, this interaction may require a specific structure that is stabilized by a number of distinct Nef domains.

Recent evidence has indicated that the heterotetrameric clathrin adaptor protein, AP-1, is the cellular trafficking factor that partners with Nef to disrupt MHC-I trafficking in HIV infected cells (8). AP-1 is composed of four subunits (β 1, γ , σ 1, μ 1A and/or μ 1B) (9-11) and is thought to promote trafficking from the *trans*-Golgi network (TGN) to endosomes (12,13). There are physically separate binding sites for cytoplasmic sorting signals on AP-1: μ 1 binds YXX ϕ signals (Y, tyrosine; ϕ , bulky hydrophobic amino acid) (14,15), the β 1 subunit (16) and a hemi-complex composed of the σ and γ subunits (17) bind dileucine motifs.

AP-1 (and Nef) co-precipitate with MHC-I in HIV-infected primary T cells (8) and siRNA to AP-1 dramatically inhibits MHC-I downmodulation caused by Nef (8,18). Nef binds AP-1 in yeast two-hybrid and GST pull-down analyses through a dileucine motif located in a solvent exposed, unstructured loop near the carboxyl terminus.

However, mutation of the dileucine motif has no effect on MHC-I down-modulation or on recruitment of AP-1 to the Nef-MHC-I complex (8). Indeed, a different binding site composed of Y₃₂₀ in HLA-A2 and a methionine at position 20 in Nef is needed for AP-1 recruitment to the Nef-MHC-I complex (8). There are currently no data indicating which AP-1 subunit(s) interacts with this novel binding site.

To better understand the formation of this complex, we performed detailed mutagenesis and binding studies. We found that Y₃₂₀ was the only amino acid in the MHC-I cytoplasmic tail absolutely required for Nef binding. In contrast, AP-1 binding to the Nef-MHC-I complex required Y₃₂₀, A₃₂₄, and D₃₂₇ in the MHC-I cytoplasmic tail. Creation of a sequence that resembled a canonical AP-1 signal (substituting YSQA₃₂₃ for YSQV₃₂₃) allowed an interaction between HLA-A2 and AP-1 that was detectable even in the absence of Nef. Additional experiments presented here indicate that the Nef acidic and polyproline domains are not absolutely required for AP-1 recruitment, but function to stabilize the interaction between AP-1 and MHC-I. Finally, we demonstrated that the natural tyrosine-binding pocket in AP-1 was necessary for Nef-induced MHC-I downmodulation and for AP-1 to bind HLA-A2. In sum, these data support the model that multiple Nef domains work together to allow Y₃₂₀ in the MHC-I cytoplasmic tail to behave as an AP-1 tyrosine signal.

Materials and Methods

Cell Culture. The Bosc packaging cell line (19) and astrocytoma cells (373MG) were cultured in DMEM supplemented with 10% FBS and 2mM penicillin, streptomycin, and glutamine. CEM T4- lymphoblastoid cell lines were cultured in RPMI 1640 that was supplemented with 10% FBS, 10mM HEPES, and 2mM penicillin, streptomycin, and Glutamine. Selection of transduced stable CEM cell lines was performed using 2.5 μ g/ml of puromycin (Fisher) or 1mg/ml of Geneticin (Gibco).

Preparation of plasmids that express the HLA-A2 cytoplasmic tail mutants.

A standard two round mutational PCR approach was utilized to generate the seven mutants. Briefly, the 5' portion of HLA-A2 was amplified from MSCV A2/Nef IRES GFP (8) using 5'*Bam*HIKozakA2 and the reverse complement of the mutant primers listed in Table 1. In a second reaction performed in parallel an overlapping 3' portion of HLA-A2 was amplified using the forward mutant primers listed in Table 1 and the reverse primer, 3'*Eco*RIstopA2. In a third reaction, the overlapping 3' and 5' fragments were annealed and amplified using 5'*Bam*HIKozakA2 and 3'*Eco*RIstopA2 primers. The construction of the HLA-A2/C chimera has been described elsewhere (20).

Preparation of plasmids that express the AP-1 μ 1 tyrosine-binding pocket mutant (F₁₇₂A/D₁₇₄S). Human AP1 μ 1A was amplified from the MegaMan human transcriptome library (Stratagene) with the forward primer 5'*Bam*HIKozakAP-1 and reverse primer named 3'*Eco*RIstopAP-1 as seen in table 1. The F₁₇₂A and D₁₇₄S mutations were made using a two round mutational PCR approach. The forward F₁₇₂A/D₁₇₄S primer was 5' F₁₇₂A/D₁₇₄S AP-1. The reverse mutant primer was the reverse complement of this sequence. An IRES PLAP cassette was generated by digesting the MSCV IRES PLAP vector with *Eco*RI and *Xho*I. AP1 μ 1A (*Bam*HI-*Eco*RI) and the IRES PLAP cassette (*Eco*RI-*Xho*I) were then ligated into MSCV Puro digested with *Bg*III to *Xho*I. This allowed for puromycin selection of stable lines and identification of F₁₇₂A/D₁₇₄S μ 1 expressing cells by flow cytometry using PLAP expression.

Preparation of plasmids that express the N-terminal alpha helix mutants of Nef. A two round mutational PCR approach similar to the A2 tail mutants was utilized to generate the nine mutants. Nef was amplified from the MSCV A2/Nef IRES GFP

previously described (8). Mutations were made in a two-step approach in which the forward primers found in the supplemental table 1 were used to amplify a fragment with reverse primer 3'*EcoRI*StopNef. At the same time, the reverse complement of the forward mutant primers plus 5'*Bam*HIKozakNef generated an overlapping fragment. A full length version of each mutant was made by then mixing the two overlapping fragments and amplifying with 5'*Bam*HIKozakNef and the reverse primer 3'*EcoRI*StopNef.

Preparation of expression plasmids containing A2/Nef fusion protein mutants. The A2/Nef fusion proteins have already been described (8). Further mutations were made to each of these constructs to add the dileucine mutation using primers previously described (8) in a two step mutational PCR approach.

DNA transfections. Transfections of MSCV or lentiviral constructs into Bosc or 293T cells were performed using *TransIT-LT1* transfection reagent (Fisher), Lipofectamine 2000 (Invitrogen), or linear polyethylenimine, MW 25,000 (Polysciences Incorporated).

Viral Transduction of CEM cell lines. Stable CEM cell lines were transduced with control or Nef-expressing Adenovirus as described previously (7). MOI for FACS analysis was 100- 500 (based on 293-cell infectivity, which is greater than CEM infectivity). MOI for immunoprecipitation and western blotting was 50-100. Transductions in low serum media ranged from 4-7 hours. Retroviral supernatants were prepared as previously described (19,21) using a bi-cistronic retroviral vector expressing an IRES GFP cassette (pMIG) (21) except that they were pseudotyped with pCMV VSV-G (Dr. Nancy Hopkins, MIT). 1×10^6 CEM T cells were spintransduced with the retroviral supernatants by centrifuging at 2500 RPM at room temperature in a tabletop centrifuge for two hours with 8 μ g/ml polybrene.

Flow Cytometry and Antibodies. 72 hours post-transductions, cells were stained in FACS buffer (PBS, 2% human serum, 1% HEPES, 1%NaN₃) with either an anti-HLA-A2 antibody (BB7.2 (22), 1:100) or anti-CD4 antibody (Serotec; 1:100) and when appropriate an antihuman placental-like alkaline phosphatase (PLAP) antibody (DAKO; 1:500). The fluorescently conjugated secondary antibody, goat anti-mouse- PE (Invitrogen, 1:250), was used for Figures 2.1, 2.3, 2.5, 2.6, and 2.7. Isotype specific,

fluorescently conjugated secondary antibodies were utilized in Figure 2.3. The secondary antibody for BB7.2 was goat anti-mouse IgG2b-specific-PE (Invitrogen; 1:250) and anti-PLAP was goat anti-mouse IgG2a-specific-PE-Cy5.5 (Caltag Laboratories; 1:500). The cells were analyzed on a Becton Dickinson FACScan apparatus using FlowJo software (Tree Star Inc.).

Biochemical transport assay, immunoprecipitation and western blot analysis.

The biochemical transport assay was performed essentially as previously described (8,23). Briefly, CEM T cells were spin transduced with retroviral supernatants as described above. Three days later 15×10^6 GFP positive cells were pulse-labeled for 30 minutes, chased in RPMI for 15 minutes and then chased in 0.5mg/ml biotin (EZ-Link sulfo-NHS-LC-Biotin, Pierce) for one hour. Lysates were immunoprecipitated for HLA-A2 with the antibody BB7.2 and eluted by boiling in 10% SDS. One-third of the immunoprecipitate was directly analyzed by SDS-PAGE while the remaining two-thirds was re-precipitated with avidin agarose (Calbiochem). Immunoprecipitation and western blot analysis was performed similarly to previous publications (7,8) with the following exceptions: A 16 hour 20mM ammonium chloride treatment was performed, the crosslinking step with DTBP was omitted, and cells were lysed with digitonin. The digitonin lysis buffer was 1% digitonin (Wako), 100mM NaCl, 50mM Tris pH 7.0, 1mM CaCl₂, and 1mM MgCl₂. PMSF and protease inhibitor tablets were included as before (8). Lysates were normalized for total protein and GFP transduction rates, when appropriate, prior to immunoprecipitation. Input controls were 1% of the immunoprecipitated protein. The wash buffer was the same as above except that it contained 0.1% digitonin. Samples were separated by SDS-PAGE and western blotted. Antibodies used were as follows: anti-Nef antibody (AG11; 1:1000, a gift from J. Hoxie, University of Pennsylvania, Philadelphia, PA; (24)), anti-AP-1 adaptin subunit, μ 1, (RY/1; 1:2,500, a generous gift from L. Traub, University of Pittsburgh, Pittsburgh, PA; (25)), anti-AP-1 adaptin subunit, γ , (BD Biosciences; IP: 1:500; WCL: 1:1000) and anti-HA (Covance; 1:1000). The secondary antibody for AG11 and anti-HA was goat anti-mouse IgG1-HRP (Zymed; 1:25-50,000), for RY/1 was rabbit anti-mouse IgG-HRP (Zymed; 1:25-50,000), and for anti-AP-1 adaptin subunit, γ , was goat anti-mouse IgG HRP (1:25-50,000). The

membranes were developed with the ECL Plus Western Blotting Detection kit from Amersham.

RNAi treatment. Duplex siRNAs (Ambion) were transfected into astrocytoma cells (373MG) as described previously (8). Briefly, on day one 1.25×10^6 373mg cells were plated onto a 100mm dish. The next day the cells were transfected using 0.64nmol duplex siRNA and 16 μ l of Lipofectamine 2000. On day three the cells were re-plated onto 6-well plates. On day four the cells were retransfected with siRNA using 0.16nmol of duplex siRNA and 4 μ l of Lipofectamine 2000. Four hours after transfection retroviral supernatants and 8 μ g/ml polybrene were added to each well. Forty-eight hours later the cells were harvested and analyzed by flow cytometry using an anti-HA antibody (1:50, Covance) or lysed for western blot analysis. The μ 1A siRNA used has been described previously (26).

5' <i>Bam</i> HIKozakA2	5'-CGGGATCCACCATGGTACCGTGCACG-3'
3' <i>Eco</i> RIstopA2	5'-GGAATTCTCACACTTTACAAGCTGTGAG-3'
5' A2 Y ₃₂₀ C	5'-GGAGGGAGCTGCTCTCAGGCT-3'
5' A2 S ₃₂₁ A	5'-GGGAGCTACGCTCAGGCTGCA-3'
5' A2 Q ₃₂₂ A	5'-AGCTACTCTGCGGCTGCAAGC-3'
5' A2 A ₃₂₃ V	5'-TACTCTCAGGTTGCAAGCAGT-3'
5' A2 A ₃₂₄ E	5'-TCTCAGGCTGAAAGCAGTGAC-3'
5' A2 S ₃₂₅ A	5'-CAGGCTGCAGCCAGTGACAGT-3'
5' A2 S ₃₂₆ A	5'-GCTGCAAGCGCTGACAGTGCC-3'
5' A2 D ₃₂₇ A	5'-GCAAGCAGTGCCAGTGCCCAG-3'
5' <i>Bam</i> HIKozakAP-1	5'-CGGGATCCACCAGGATGTCCGCCAGCGCCGTCTACGTG-3'
3' <i>Eco</i> RIstopAP-1	5'-CGGAATTCTCACTGGGTCCGGAGCTGGTAATC-3'
5' F ₁₇₂ A/D ₁₇₄ S AP-1	5'-CGGAAGAATGAGGTGCGCCCTCTCCGTGATCGAGTCTGTCAAC-3'
5' <i>Bam</i> HIKozakNef	5'-CGGGATCCGCCACCATGGGTGGCAAGTGGTCAAAAA-3'
3' <i>Eco</i> RIStopNef	5'-GCGAATTCTCAGCAGTTCTTGAAGTACTC-3'
5' Nef R ₁₇ A	5'-CCTGCTGTAGCGGAAAGAATG-3'
5' Nef E ₁₈ A	5'-GCTGTAAGGGCAAGAATGAGA-3'
5' Nef R ₁₉ A	5'-GTAAGGGAAGCAATGAGACGA-3'
5' Nef R ₂₁ A	5'-GAAAGAATGGCACGAGCTGAG-3'
5' Nef R ₂₂ A	5'-AGAATGAGAGCAGCTGAGCCA-3'
5' Nef A ₂₃ V	5'-ATGAGACGAGTTGAGCCAGCA-3'
5' Nef E ₂₄ A	5'-AGACGAGCTGCGCCAGCAGCA-3'
5' Nef P ₂₅ A	5'-CGAGCTGAGGCAGCAGCAGAT-3'
5' Nef A ₂₆ V	5'-GCTGAGCCAGTAGCAGATGGG-3'

Primer sequences utilized to mutate the HLA-A2 cytoplasmic tail, the AP-1 μ 1 tyrosine-binding pocket, and Nef's α -helix. The reverse complement of each mutational primer was also utilized to introduce the appropriate mutations.

Table 2.1. Primer Name and Primer Sequence

Results

Mutating three amino acids in the cytoplasmic tail of HLA-A2 abrogated Nef-induced downmodulation and AP-1 recruitment. The HIV Nef protein binds to the MHC-I cytoplasmic tail and recruits AP-1. However it is not clear whether AP-1 is linked to MHC-I via the Nef protein or whether there are contacts amongst all three proteins. The sequences of the HLA-A, B, C, and E cytoplasmic tails and the amino acids that differ between Nef-responsive and unresponsive molecules are shown in Figure 2.1A. To elucidate the requirements for the formation of this complex, we performed alanine-scanning mutagenesis along the domain of MHC-I HLA-A2 that is needed for responsiveness to Nef (27) (bolded region in Figure 2.1A). In addition, Y₃₂₀ was mutated to cysteine to mimic the HLA-C molecule and A₃₂₄ was mutated to glutamate to mimic the HLA-E molecule. We also utilized a construct in which the entire HLA-C cytoplasmic tail was fused to the HLA-A2 extracellular and transmembrane domains (20). Stable lines were made in CEM T cells and each line was transduced with either control or Nef-expressing adenovirus. Surface expression of each HLA-A2 variant and endogenous CD4 was measured by flow cytometry as shown in Figures 2.1B and C. The endogenous CD4 stain was utilized to show that Nef was indeed being expressed and functioning inside the cell, even when the HLA-A2 variant was not affected by Nef expression. Consistent with previously published data, HLA-A2 with the C tail (A2/C) and the HLA-A2 mutants Y₃₂₀A, Y₃₂₀C, A₃₂₄E and D₃₂₇A were all unaffected by Nef expression. Partial downmodulation was observed when amino acids Q₃₂₂, S₃₂₅, and S₃₂₆ were mutated (Figures 2.1B and C). Interestingly as seen in Figure 2.1D, the mutation A₃₂₃V caused a significant decrease in steady state surface expression (p -value = 0.007) and even further decreased the surface expression of HLAA2 in Nef expressing cells (p -value = 0.01). This substitution creates a sequence more similar to a canonical AP-1 binding motif (YXX ϕ), and thus, may allow AP-1 to interact with MHC-I even in the absence of Nef.

To determine the mechanism for the variation in responsiveness to Nef caused by these sequence substitutions, we immunoprecipitated each HLAA2 mutant and assayed

for co-precipitation of Nef and AP-1. For these analyses, we used a newly developed protocol that utilized digitonin lysis and wash buffers rather than our previously published protocol, which required protein cross-linking (8). As shown in Figure 2.2A, Nef and AP-1 (μ and γ subunits) readily co-precipitated with wild type HLA-A2, (lanes 1, 5 and 11) but not with A2/C (lanes 10 and 16). In accordance with the FACS analysis, the HLA-A2 mutants Y₃₂₀A, Y₃₂₀C, A₃₂₄E, and D₃₂₇A, which were not downmodulated by Nef, did not recruit the μ 1 or γ subunits of AP-1 (Figure 2.2A, lanes 2, 3, 12, and 15). Interestingly, Nef was still able to bind Y₃₂₀C, A₃₂₄E, and D₃₂₇A in the absence of AP-1 (Figure 2.2A, lanes 3, 12, and 15). These results indicate that the MHC-I sequence requirements for Nef and AP-1 binding are separable and are thus likely to be independent events. In agreement with previously published results (8), only the Y₃₂₀A mutation did not bind either Nef or AP-1 (Figure 2.2A, lane 2). Additional mutations (S₃₂₁A, Q₃₂₂A, S₃₂₅A and S₃₂₆A) all bound Nef and recruited AP-1 as well as or better than wild type HLA-A2 (Figure 2.2A, lanes 6, 7, 13 and 14).

Perhaps because of the increased hydrophobicity of valine compared to alanine, the HLA-A2 mutant A₃₂₃V bound AP-1 and Nef much better than wild type HLA-A2 (compare lane 5 to lane 9). Interestingly, even in the absence of Nef we observed that HLA-A2 A₃₂₃V co-precipitated AP-1 (Figure 2.2B, compare 4d with 8d), but Nef dramatically enhanced this interaction (Figure 2.2B, compare 8d and 9d). The combination of the need for Y₃₂₀ and the enhanced interaction with A₃₂₃V suggested that Nef recruited AP-1 by utilizing the canonical YXX ϕ signal-binding pocket found in the AP-1 μ 1 subunit.

The μ 1 subunit of AP-1 uses its canonical tyrosine-binding pocket for Nef-induced downmodulation of HLA-A2. To determine whether Nef recruited AP-1 to bind the YSQA sequence in HLA-A2 via its YXX ϕ signal binding pocket, we utilized data generated from the study of a similar adaptor protein complex (AP-2). Prior studies have found that AP-2 behaves as a dominant negative when specific mutations (F₁₇₄A and D₁₇₆S) in its tyrosine-binding pocket are made (28,29). Similarities between AP-1 and AP-2 led us to hypothesize that the analogous mutation in AP-1 μ 1 would similarly act as a dominant negative inhibitor of Nef function if the tyrosine binding pocket was required for this Nef activity. To test this, we made the tyrosine-binding pocket mutant

(TBPM) in the $\mu 1$ subunit and expressed it or the wild type together with the reporter gene PLAP driven off of an internal ribosomal entry site (IRES). We then used flow cytometry to demonstrate that the $\mu 1$ TBPM indeed acted as a dominant negative inhibitor of Nef (Figure 2.3A, compare PLAP⁻ to PLAP⁺ in the lower two FACS plots). This effect, which was quantified in Figure 2.3B, was highly significant (p -value = 0.001). To confirm that this mutant acted as a dominant negative inhibitor because it failed to bind the YSQA sequence in HLA-A2, we performed the IP-western blot experiment shown in Figure 2.3C. In agreement with the flow cytometric results, $\mu 1$ TBPM expression resulted in a decrease in the amount of AP-1 $\mu 1$ and $\gamma 1$ that was able to immunoprecipitate with HLA-A2 and Nef (compare lanes 2 and 6). Interestingly, we also consistently observed that Nef binding was diminished in the samples expressing $\mu 1$ TBPM. These data indicate that, even though Nef can bind the cytoplasmic domain without AP-1 (Figure 2.2A e.g. Y₃₂₀C mutant), the presence of AP-1 plays a role in stabilizing Nef binding to HLA-A2.

The methionine at position 20 in Nef is the only amino acid in the N-terminal α -helical domain needed for A2 down-modulation. To further understand how Nef is involved in MHC-I and AP-1 binding, we examined the charged α -helical domain from amino acids 17 through 26 in Nef in more detail. This region, as well as the methionine at position 20 within it, have been shown to be required for Nef binding to HLA-A2, and AP-1 recruitment (7,8). To determine whether any other amino acids in this domain contributed, we performed alanine-scanning mutagenesis of this domain (Figure 2.4A) and transiently expressed each Nef mutant in CEM T cells using a murine retroviral vector that also expressed GFP. As shown in Figure 2.4B, we found that only M₂₀ was needed for HLA-A2 down-modulation. In comparison, none of the α -helical mutants were defective at CD4 down-modulation. All of the Nef mutants, including M₂₀A, were expressed in transduced cells at least as well as wild type Nef based on western blot analysis (Figure 2.4C).

Nef's acidic (E₆₂₋₆₅) and polyproline domains (P_{75/78}) stabilize the interaction between the A2/Nef fusion protein and AP-1. Previously published data indicated that all the domains in Nef that are required for MHC-I down-modulation are also required for Nef to bind the cytoplasmic tail of MHC-I (7). To determine whether these domains also

contributed to AP-1 recruitment, Roeth *et al* utilized an A2/Nef fusion protein in which Nef was fused to the end of the cytoplasmic domain in order to bypass the requirement for Nef binding (Figure 2.5A) (8). This analysis revealed that both M₂₀A in Nef and the tyrosine residue in the MHCI cytoplasmic tail are necessary for AP-1 recruitment. Three additional regions in Nef are also needed for MHC-I down-modulation (30,31): an acidic region (E₆₂₋₆₅), an aspartate residue (D₁₂₃), and a polyproline repeat (P_{72/75/78}). To further examine the roles of these domains, we used the fusion proteins containing these mutations and performed FACS analysis. In contrast to what was observed with *trans*-Nef we observed little effect of mutating these domains when Nef was in *cis* (Figure 2.5B and C). A partial explanation may be that expression of some of these mutants was slightly lower than wild type A2/Nef (Figure 2.5D). However, the defect in AP-1 binding shown in Figure 2.5E, for M₂₀A in particular (compare lanes 2 and 3), was far greater than the differences in protein expression shown in Figure 2.5D.

Surprisingly, in contrast to our previously published results, we consistently observed that mutation of the acidic (E₆₂₋₆₅) and polyproline (P_{75/78}) domains also disrupted AP-1 binding (Figure 2.5E, lanes 4 and 5); although, these domains were not required to the same extent as M₂₀. The difference between these experiments and our previously published results (8), was a change in protocol in which the immunoprecipitation experiments were performed without protein crosslinker, which we found was dispensable when digitonin was used as the detergent in our lysis and wash buffers. Thus, these domains likely perform a stabilizing function that the addition of crosslinker replaced.

The A2/Nef fusion protein contains two active trafficking signals. As shown in Figure 2.5, mutating M₂₀ in the A2/Nef fusion protein abrogated AP-1 recruitment (Figure 2.5E lane 3), but, unlike *trans*-Nef, the mutant A2/Nef fusion protein was still down-modulated relative to wild type HLA-A2 (Figure 2.5B and C). To further explore this apparent enigma, we asked whether another trafficking signal might be active in the fusion protein. As discussed above, Nef also contains a dileucine motif, which is necessary to disrupt the trafficking of other Nef targets, but which is normally dispensable for Nef's effects on MHC-I (8). Surprisingly, we found that mutating the dileucine motif in the context of the fusion protein (A2/xLL Nef) reduced down-

modulation by about 40-50% as seen by flow cytometry (Figure 2.6A and C and quantified in Figure 2.6B and D respectively). Additionally, the double mutant, which lacked both the dileucine and M₂₀ (A2/M₂₀AxLLNef) [or the entire α helical domain, including M₂₀ (A2/V₁₀E Δ 17-26xLL)], was even more defective, retaining only about 10% of wild type activity (Figure 2.6A and B). Similarly, the individual mutation of Y₃₂₀ partially inhibited Nef activity (Figure 2.6C and D), whereas the combined mutation of both Y₃₂₀ and dileucine completely abrogated Nef activity (Figure 2.6C and D). Thus, in the fusion protein there are two trafficking signals; one that depends on Y₃₂₀ in MHC-I and M₂₀ in Nef and the other which depends on the dileucine motif. Both signals need to be removed to abrogate the effects of Nef on the fusion protein.

Additional double mutant fusion proteins, which lacked the acidic, and polyproline domains in addition to the dileucine motif had intermediate phenotypes, demonstrating a partial requirement for these domains in the fusion protein (Figure 2.6A and B). The oligomerization domain (D₁₂₃) was absolutely required whether or not the dileucine motif was present (Figures 2.5 B and C and Figure 2.6 A and B).

The pattern of AP-1 recruitment for doubly mutated fusion proteins shown in Figure 2.6E perfectly matched the singly mutated fusion protein recruitment pattern seen in Figure 2.5E and correlated well with HLA-A2 down-modulation (Figure 2.6B). Thus, it was the presence of the dileucine motif in the fusion proteins, which masked a requirement for these domains in Figure 2.5B and C. Finally, we also confirmed previously published results (8) that co-precipitation of A2/Nef with AP-1 was independent of the dileucine motif (Figure 2.6E, compare lanes 2 and 6). Thus, the activity of the dileucine motif as a trafficking signal in the fusion protein, was not due to its ability to bind AP-1.

To further examine the role of AP-1 in tyrosine and dileucine-dependent trafficking of the fusion protein, we knocked down AP-1 expression with siRNA directed against the μ 1A subunit (Figure 2.7A). As shown in Figure 2.7B and C, there was a significant effect of siRNA to AP-1 on trafficking of the fusion protein. This effect was observed most dramatically when the dileucine motif was mutated (A2/xLL in Figures 2.7B and C, $p=0.008$). In contrast, when Y₃₂₀ was mutated (Y₃₂₀A/Nef), the addition of siRNA to AP-1 did not significantly affect down-modulation due solely to the effects of

the dileucine motif. These data are consistent with the fact that the dileucine motif was not needed for AP-1 binding by the fusion protein (Figure 2.6E) and indicate that the dileucine motif likely interacts with another cellular trafficking factor.

Nef has been shown to affect the transport of MHC-I to the cell surface (32) as well as the internalization of MHC-I from the cell surface (1), and therefore, we examined these pathways using the fusion proteins. As a control, a protein that was similar in size to Nef (YFP), was fused to the HLA-A2 tail (A2-YFP). Compared to wild type HLA-A2, A2-YFP was exported to the cell surface at a somewhat slower rate (Figure 2.7D). However, wild type Nef disrupted export much more efficiently. The effect of wild type Nef was dependent on the presence of both the dileucine and the tyrosine motifs within the fusion protein. When both domains were mutated, the fusion protein was transported to the cell surface to the same degree as the A2/YFP fusion protein (Figure 2.7D).

Finally, we also asked whether both motifs affected the rate of internalization of the fusion protein. As shown in Figure 2.7E, the fusion protein (A2/Nef) was internalized substantially more rapidly than HLA-A2 alone. Mutation of each motif partially reversed this acceleration of internalization. However, it was necessary to mutate both signals (A2Y₃₂₀A/L_{164/165}A) to completely reverse the effects of Nef. Thus, in the context of the fusion proteins, the tyrosine and dileucine motifs had redundant trafficking functions. However, only the tyrosine-based signal co-precipitated AP-1 and was dependent on AP-1 expression.

Discussion

In sum, we present evidence that Nef binding to MHC-I resulted in recruitment of AP-1 to the MHC-I cytoplasmic tail and that recruitment of AP-1 required the natural tyrosine-binding pocket in the AP-1 μ 1 subunit. Interestingly, AP-1 binding to the MHC-I cytoplasmic tail tyrosine required additional amino acids (Y₃₂₀XXAA₃₂₄XXD₃₂₇) beyond the usual YXX ϕ signal. The Nef protein stabilized this unusual interaction via several domains. A methionine within Nef (M₂₀) was absolutely required. Additionally, Nef's acidic (E₆₂₋₆₅) and polyproline (P_{75/78}) domains had a stabilizing effect on AP-1 binding that was apparent when digitonin buffer was used and protein cross-linker was omitted. We observed that Nef was able to bind the HLA-A2 tail in the absence of detectable AP-1. However, experiments using μ 1 TBPM provided evidence that the presence of wild type AP-1 was able to enhance the interaction amongst the three proteins. Finally, we demonstrated that fusion of Nef to the HLA-A2 cytoplasmic tail activated a second trafficking signal composed of Nef's dileucine motif, which does not normally affect MHC-I trafficking. The activity of this second signal did not require AP-1 expression.

The AP-1 signal in the MHC-I cytosolic domain. Previous studies had revealed that Y₃₂₀, A₃₂₄, and D₃₂₇ in the HLA-A2 tail are required for Nef-induced MHC-I down-modulation (5,6). Here we have demonstrated that each of these amino acids was also required for AP-1 recruitment. It is intriguing that only the amino acids unique to HLA-A and B, but missing from HLA-C and HLA-E were needed for this interaction. Based on these results, it is tempting to speculate that AP-1 might normally bind a subset of MHC-I molecules under certain conditions. For example, it may be important to alter the trafficking of MHC-I molecules into the endolysosomal pathway in myeloid cell types for cross-presentation of exogenous antigens (33).

We also demonstrated here that changing YSQA₃₂₃ to YSQV₃₂₃ caused a decrease in HLA-A2 surface expression and an increase in AP-1 recruitment in the absence of Nef. This mutation, which results in a sequence that more closely resembles a YXX ϕ signal, also bound Nef better and resulted in more Nef-dependent downmodulation. These data confirmed prior reports that mutating this region to YSQL₃₂₃ decreases the surface stability of HLA-A2, causes an accumulation in the *trans*-Golgi network, and increases

Nef responsiveness (34). In sum, these results suggest that the MHC-I cytoplasmic tail contains a region that resembles an AP-1 binding site and supports the notion that AP-1 might be utilizing its natural tyrosine-binding pocket in the $\mu 1$ subunit, which interacts with YXX ϕ signals in cargo proteins (14,15).

Domains of AP-1 involved in formation of the Nef/MHC-I/AP-1 complex. To examine which AP-1 domains were involved in binding to MHC-I and/or Nef, we used a dominant negative $\mu 1$ subunit (TBPM). TBPM contained two amino acid substitutions in the tyrosine-binding pocket and behaved as a dominant negative inhibitor of AP-1 binding to the Nef-MHC-I complex. These data provide substantial evidence that the AP-1 $\mu 1$ tyrosine-binding pocket is necessary for formation of the Nef-MHC-I-AP-1 complex. Interestingly, in the presence of AP-1 $\mu 1$ TBPM, we also noted a decrease in Nef binding to HLA-A2. This suggests the possibility that the presence of AP-1 also stabilized Nef binding to the complex.

The Nef binding site in the MHC-I cytoplasmic tail. Surprisingly, we found that the Y₃₂₀A mutation in the cytoplasmic tail also disrupted Nef binding and that other individual point mutations in this region had no effect on the ability of Nef to co-immunoprecipitate with HLA-A2. Despite these results, it is clear that Nef has contacts with other amino acids in the cytoplasmic tail because Nef fails to bind to the HLA-E cytoplasmic tail (27), even though it has a tyrosine at position 320. In addition, certain serine to alanine substitutions in the HLA-A2 cytoplasmic tail enhance Nef binding (35). Based on a number of experiments, prior studies had concluded that enhanced binding to these serine-to-alanine substitutions reflected a preference for Nef to bind immature forms of MHC-I, which are hypophosphorylated (35).

Finally, data presented here indicate that Nef can bind MHC-I independently of AP-1. These data are consistent with prior reports that purified Nef protein directly interacts with a purified HLA-A2 cytoplasmic tail-GST fusion protein (27). Despite the fact that Nef can bind to MHC-I in the absence of AP-1, it remains possible that AP-1 can further stabilize this interaction as suggested by the reduced Nef binding we observed when AP-1 TBPM was expressed.

Domains of Nef involved in formation of the Nef/MHC-I/AP-1 complex. Previous studies had indicated that all of the Nef domains known to be required for

MHC-I down-modulation were also required for Nef binding to MHC-I (7). Additional experiments, which bi-passed this step by directly fusing Nef to the cytoplasmic tail domain, revealed that a region of the amino terminal α -helical domain (and M₂₀ within this domain), was also required for AP-1 recruitment to the Nef-MHC-I complex (8). Using new assay conditions, which omitted protein crosslinker, we were able to also detect a role for the acidic domain (E₆₂₋₆₅) and more dramatically, for the polyproline (P_{75/78}) domain in AP-1 recruitment. Because these domains were not absolutely required for AP-1 recruitment and because they were not needed in the presence of crosslinker (8), it is likely that they played a stabilizing role.

Additional studies will be needed to more precisely understand the role of each amino acid domain in the overall structure of the Nef-MHC-I-AP-1 complex. For example, it is unclear how it is possible for MHC-I Y₃₂₀ to be required for both Nef and AP-1 recruitment. Moreover, it is unclear as to why cysteine substitutions at position 320 in the MHC-I cytoplasmic tail support Nef, but not AP-1 binding. Possible explanations for these data are presented in Figure 2.8. First, Y₃₂₀ may be buried in the tyrosine-binding pocket of AP-1 and may not directly interact with Nef. In this scenario, Y₃₂₀ may be needed to maintain the conformation of the MHC-I tail that Nef binds (Figure 2.8A). When cysteine is substituted at position 320, the structure of the tail is maintained, supporting Nef binding, but the cysteine is unable to interact with the tyrosine-binding pocket and so this mutant fails to recruit AP-1 (Figure 2.8A). Another possible explanation for how Y₃₂₀ might interact with both proteins is that a conformational change occurs which allows sequential interactions to occur (Figure 2.8B).

The role of Nef's dileucine motif. Finally, we demonstrated that fusing Nef to the cytoplasmic tail of MHC-I activated a dileucine motif in Nef to behave as a trafficking signal. Under normal circumstances, Nef's dileucine motif is needed to disrupt the trafficking of CD4, but is not needed for down-modulation of HLA-A2 (8,36). The explanation for these findings may be that the dileucine motif may normally be hidden when Nef is bound to MHC-I (Figure 2.8), but becomes activated when Nef binds CD4. The conformation of Nef in the fusion protein appears to be such that both signals are active.

The dileucine motif and the tyrosine-based trafficking signal in MHC-I that forms upon Nef binding behaved similarly in that both affected protein export and internalization. However, only the tyrosine-based signal required AP-1 expression for activity. This is consistent with work by other groups, which have suggested that the dileucine motif binds to AP-2 (20).

Interestingly, we also observed that the relative ability of Nef to affect the internalization of MHC-I was greater with the fusion protein than with *trans*-Nef (35). This observation supports the model that Nef does not normally promote internalization of surface MHC-I because phosphorylation of the mature MHC-I cytoplasmic tail domains limits Nef binding (35). It is possible that continued expression of “old” MHC-I molecules presenting cellular epitopes at the time of viral infection benefits the virus by providing some protection from recognition and lysis by natural killer cells (35).

In sum, our studies shed further light on the mechanism by which Nef downmodulates MHC-I expression to promote viral immune evasion. Our work supports a model in which Nef stabilizes the interaction of a tyrosine in the MHC-I cytoplasmic tail with the natural tyrosine-binding pocket of AP-1. The observation that the dileucine motif in Nef can affect MHC-I trafficking only when Nef is fused to the MHC-I cytoplasmic tail supports the concept that Nef takes on notably different structural forms in different contexts, revealing or obscuring trafficking signals as needed. A greater understanding of the interactions amongst these proteins will facilitate the development of pharmaceuticals, which may someday help combat AIDS.

Figure 2.1. Three amino acids in the HLA-A2 tail are required for Nef-induced down-modulation. (A) Sequence of the HLA-A, B, C and E cytoplasmic tails. The amino acids that differ between classical (HLA-A and HLA-B) and non-classical (HLA-C and HLA-E) MHC class I molecules are underlined. (B) Nef-induced down-modulation of HLA-A2 and cytoplasmic tail mutants. Stable CEM cell lines were made and transduced with a control adenovirus or an adenovirus that expressed HIV-1 Nef as described in Materials and Methods. The cells were stained 72 hours post-transduction for HLA-A2 surface expression (left column) or endogenous CD4 surface expression (right column); shaded curve, control adenovirus; black line, Nef-expressing adenovirus. (C) Quantitation of Nef-induced down-modulation on various HLA-A2 tail mutants and HLA-A2/C. The mean fold down-modulation +/- standard deviation is shown for three independent experiments. (D) Quantitation of FL-2 mean fluorescence in the absence or presence of Nef. Gray bars indicate control adenovirus and white bars indicate Nef adenovirus treated cells. The FL-2 mean fluorescence, standard deviation, and paired T-Tests were calculated from three independent experiments.

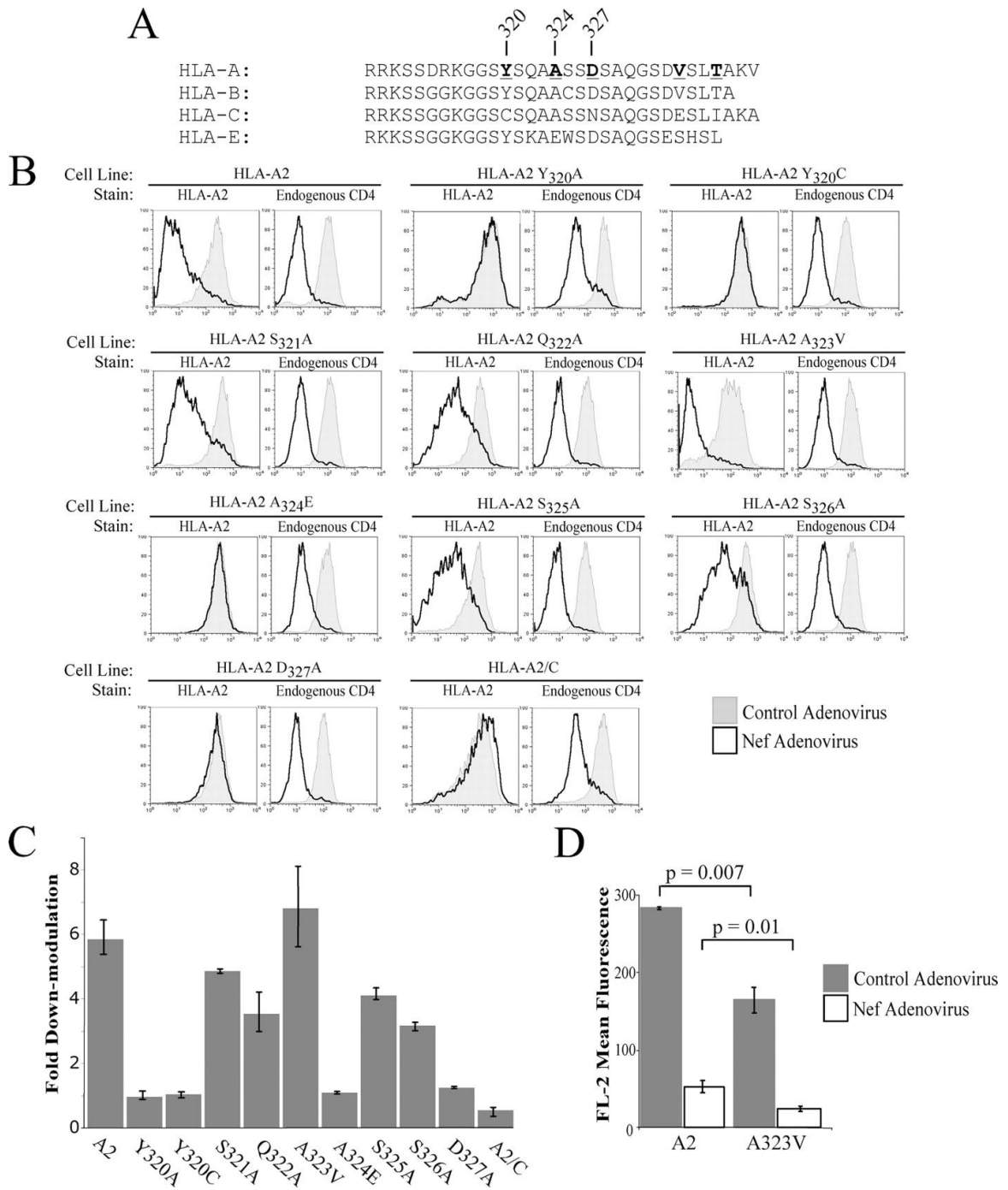


Figure 2.1. Three amino acids in the HLA-A2 tail are required for Nef-induced down-modulation.

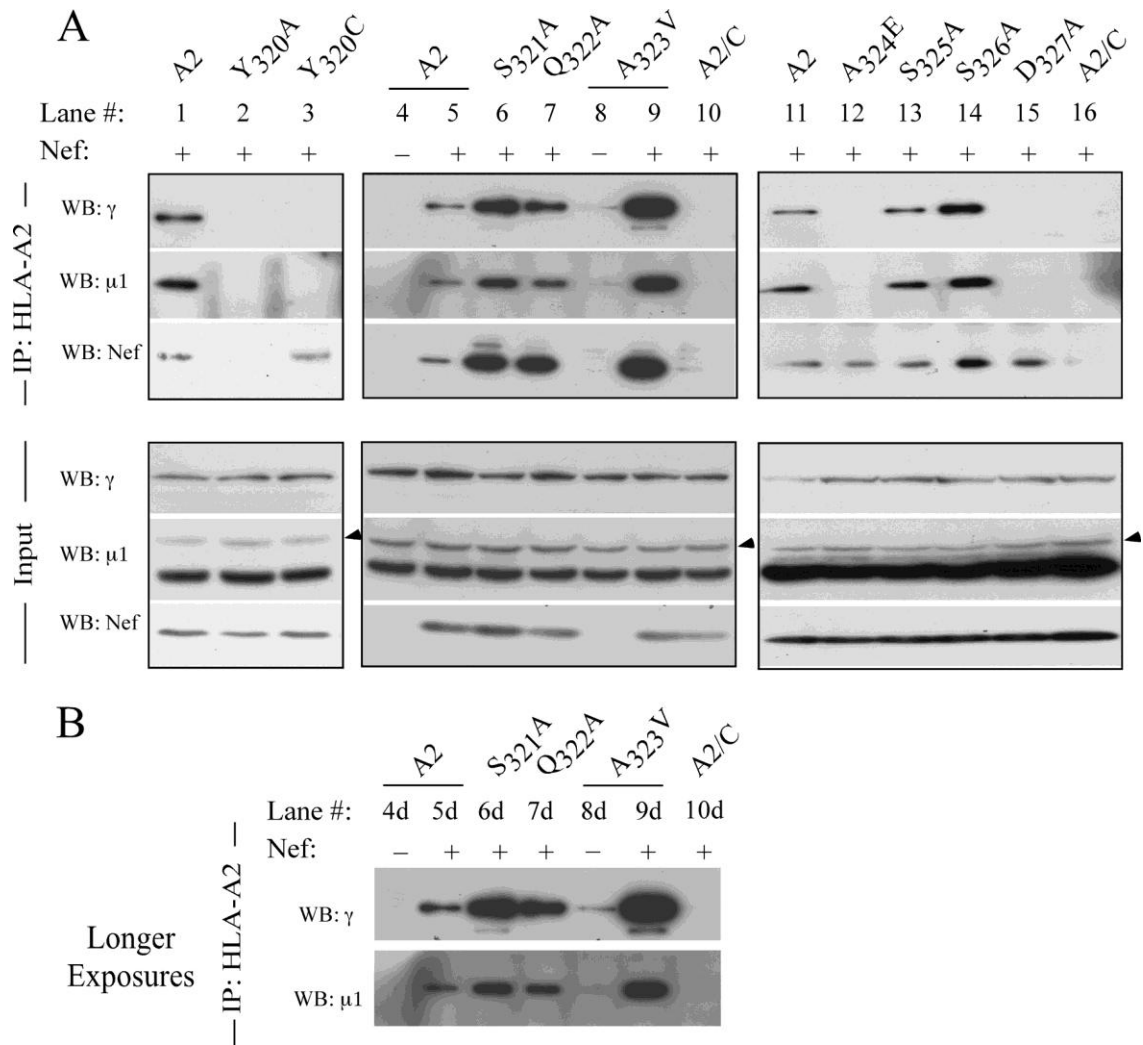


Figure 2.2. Three amino acids in the HLA-A2 cytoplasmic tail are required for AP-1 recruitment.

(A) Stable cell lines expressing wild type HLA-A2 or the various HLA-A2 tail mutants were transduced with either a control or a Nef-expressing adenovirus. The cells were harvested, lysed, and immunoprecipitated with the anti-HLA-A2 antibody, BB7.2 as described in Materials and Methods. Proteins that stably interacted with HLA-A2 were detected by western blotting as indicated. Relative protein levels prior to immunoprecipitation are shown as input. Results are representative of at least three independent experiments. (B) AP-1 interacted with A₃₂₃V in the absence of Nef. Longer exposures of the western blot panels shown in part A.

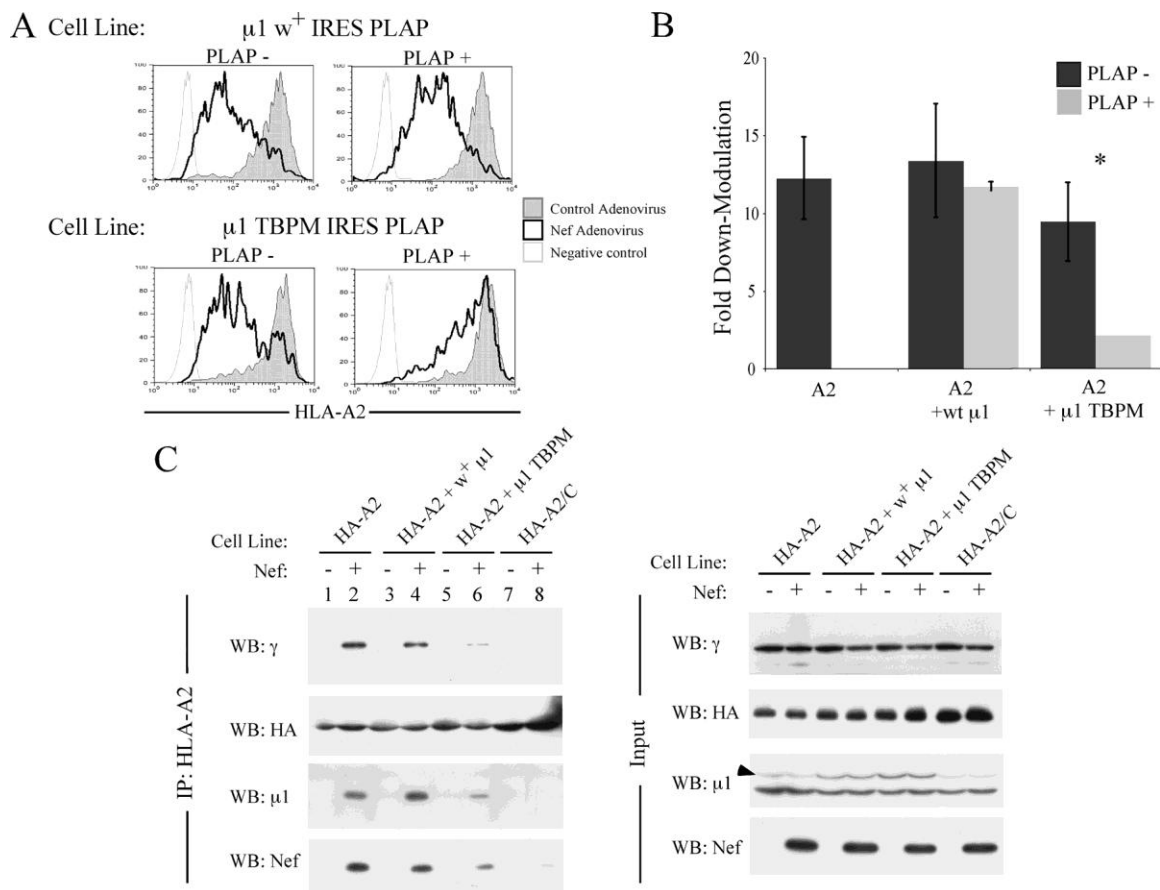


Figure 2.3. The tyrosine-binding pocket in AP-1 $\mu 1$ is necessary for Nef-induced HLA-A2 downmodulation.

(A) Expression of F₁₇₂A/D₁₇₄S AP-1 $\mu 1$ (TBPM) reverses Nef-induced down-modulation of HLA-A2. CEM cells stably expressing HA-HLA-A2 were first transduced with a bicistronic murineretroviral vector expressing PLAP and either a wild type or TBPM AP-1 $\mu 1$. Stable cell lines were selected with puromycin. These cells were then transduced with either control or Nef-expressing adenovirus. Three days after this transduction, flow-cytometric analyses were performed to examine HLA-A2 surface expression. (B) Quantitation of Nef-induced down-modulation in the presence of AP-1 $\mu 1$ TBPM. Calculations were based on the medians for HLA-A2 fluorescence. The mean fold downmodulation +/- standard deviation is shown. * *P*-value for paired T-Test was 0.001. (n=6) (C) Expression of $\mu 1$ TBPM decreases recruitment of AP-1 $\mu 1$ and μ subunits to the HLA-A2 cytoplasmic tail. Cells that stably expressed HA-HLA-A2 and either a wild type form of AP-1 $\mu 1$ or TBPM $\mu 1$ were immunoprecipitated and proteins interacting with A2 were imaged through western blot analysis. Results were similar in three independent experiments.

Figure 2.4. The methionine at position 20 is the only necessary amino acid in the 17–26 region of Nef. (A) Amino acid sequence of the region in the amino-terminal α -helix previously shown to be necessary for Nef-mediated MHC-I down-modulation. (B) Flow cytometric analysis of down-modulation of HLA-A2 by various Nef mutants. CEM HA-HLA-A2 cells were transduced with a bicistronic murine retroviral vector expressing the indicated mutant and GFP. The GFP positive population is shown; shaded curve, empty vector; black line, Nef variant. (C) Western blot (WB) of Nef expression levels.

A

Nef α -helix:	RERMRRAEPA
R ₁₇ A:	A ERMRRAEPA
E ₁₈ A:	R A ERMRRAEPA
R ₁₉ A:	RE A ERMRRAEPA
M ₂₀ A:	RER A RRAEPA
R ₂₁ A:	RERM A RAEPA
R ₂₂ A:	RERMRA A EPA
A ₂₃ V:	RERMRR V EPA
E ₂₄ A:	RERMRR A PA
P ₂₅ A:	RERMRR A EA
A ₂₆ V:	RERMRR A EP V

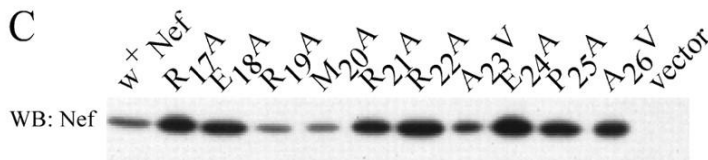
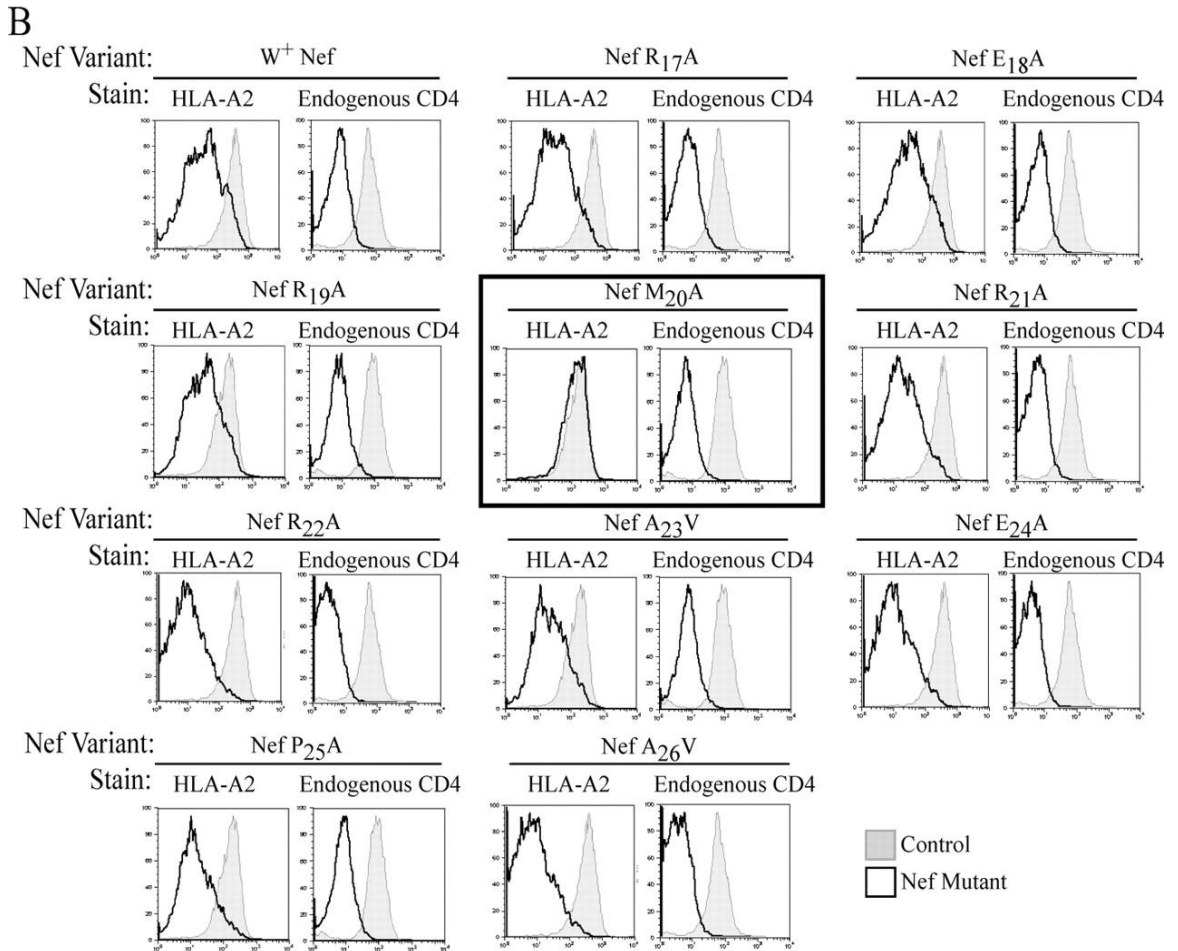


Figure 2.4. The methionine at position 20 is the only necessary amino acid in the 17–26 region of Nef.

Figure 2.5. The role of Nef M₂₀, acidic, and SH3 binding domains in AP-1 recruitment. (A) Schematic diagram of the A2/Nef fusion protein. TM, transmembrane domain. (B) Flow cytometric analysis of A2/Nef mutants. CEM SS cells were transduced with a bicistronic murine retroviral vector expressing GFP plus either HLA-A2 or the indicated A2/Nef fusion protein. The GFP-positive population is shown; shaded curve, HLA-A2 only; black line, A2/Nef variant. (C) The mean % wild type activity \pm S.D. is shown. (n = 3). (D) Steady state levels of each fusion protein normalized for protein concentration and transduction efficiency. (E) AP-1 recruitment by A2/Nef mutants. Transduced cells were immunoprecipitated (IP) for HLA-A2, and Western blot (WB) analysis was performed as described under “Materials and Methods.” Similar results were obtained in three independent experiments.

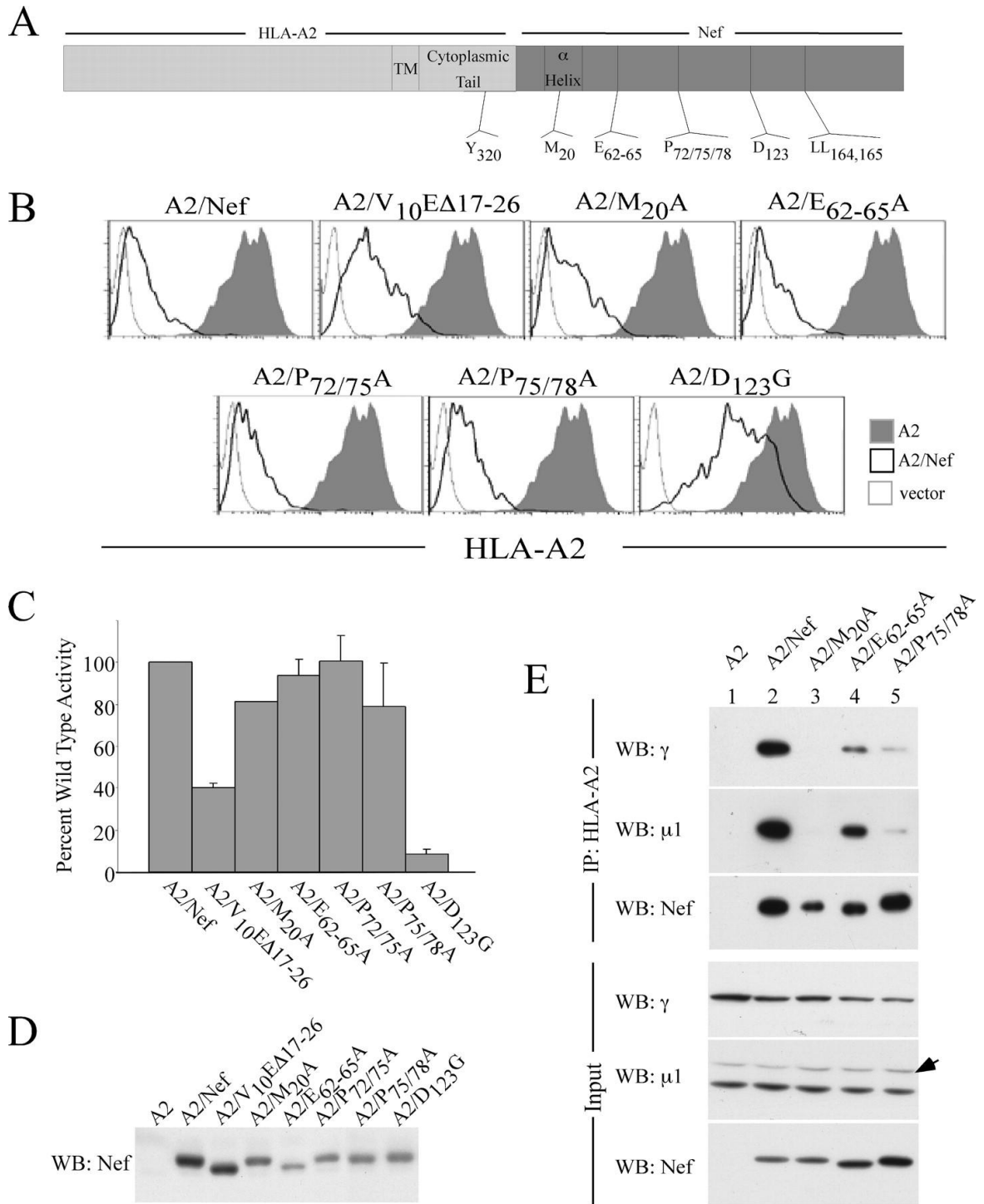


Figure 2.5. The role of Nef M₂₀, acidic, and SH3 binding domains in AP-1 recruitment.

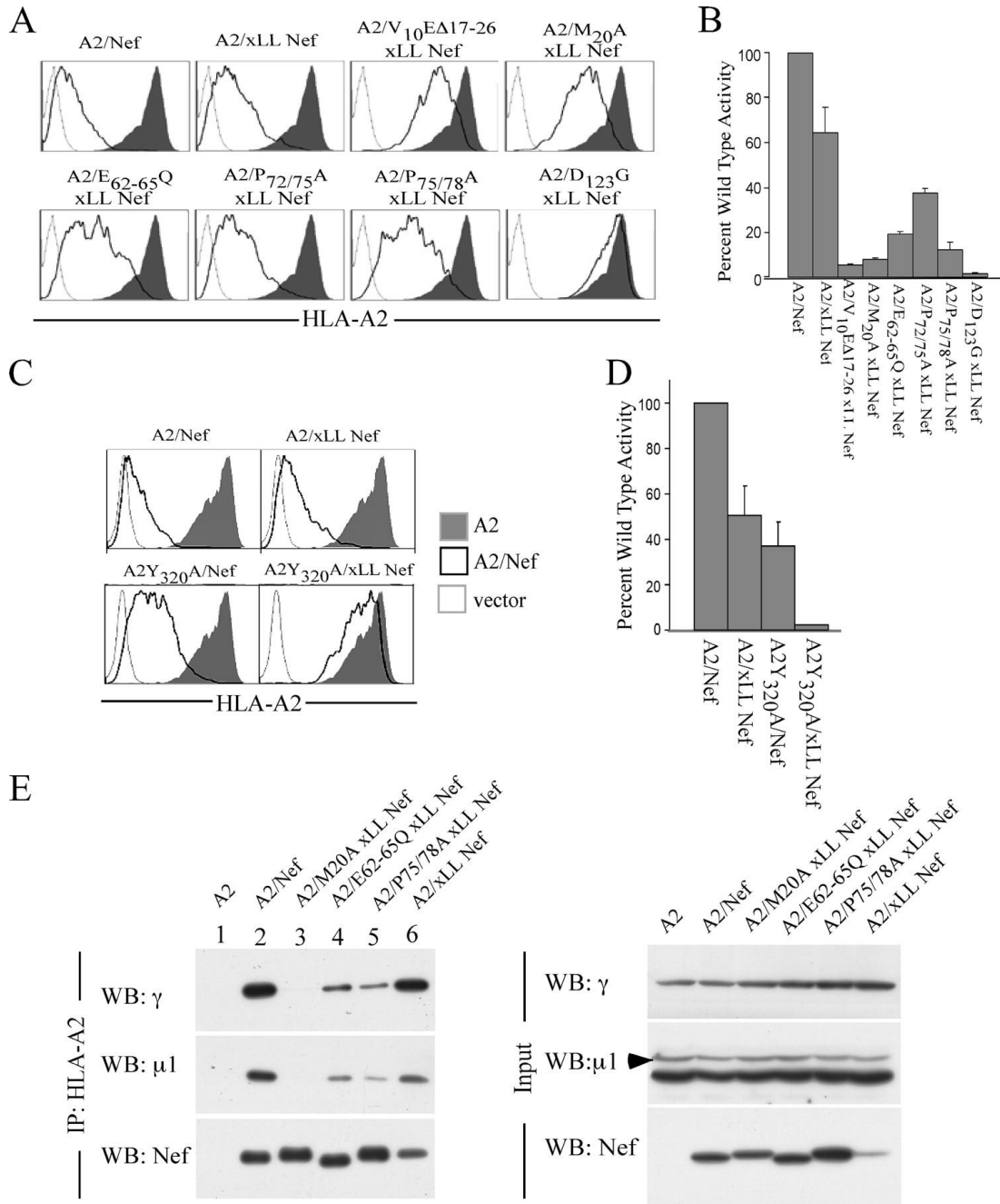


Figure 2.6. In addition to the AP-1 binding site, a second site, the dileucine motif, functioned as trafficking signal in the A2/Nef fusion protein.

(A and C) Flow cytometric analysis of various A2/Nef mutants. CEM SS cells were transduced as in Fig. 2.5; shaded curve, A2 only; black line, A2/Nef variant. (B and D) Quantitation of down-modulation. The mean \pm S.D. is shown ($n = 8$ and 6 respectively). (E) AP-1 recruitment to A2/Nef double mutants. Immunoprecipitation (IP) and Western blot (WB) analysis was performed as in Fig. 2.5B. Similar results were obtained in two independent experiments.

Figure 2.7. Trafficking signals within the A2/Nef fusion differ with regard to AP-1 dependence. (A) Western blot (WB) analysis of AP-1 expression levels in 373MG astrocytoma cells transfected with siRNA directed against AP-1 μ l. (B) siRNA directed against AP-1 affects the downmodulation of A2/Nef fusion protein variants only when Y₃₂₀ is present. 373MG astrocytoma cells were transduced as in Fig. 2.5B. Shaded curve, A2 only; black line, A2/Nef variant; gray line, A2 negative. (C) Quantitation of fold decrease in surface expression. The mean \pm S.D. is shown (n = 4). Significant differences are denoted with an asterisk. *p* values for paired t test; A2/Nef, 0.0086; A2/xLL, 0.0080. (D) Transport assay. CEM SS cells were transduced as described in Fig. 2.5B. A transport assay using a cell-impermeable biotinylation reagent to identify surface protein was performed as described under “Materials and Methods.” The mean fraction of total HLA-A2 arriving at the cell surface in 1 h is indicated \pm S.D. (n = 4). (E) Internalization assay. CEM SS cells were transduced as described in Fig. 2.5B. A FACS-based internalization assay was performed as described previously (32) (n = 2).

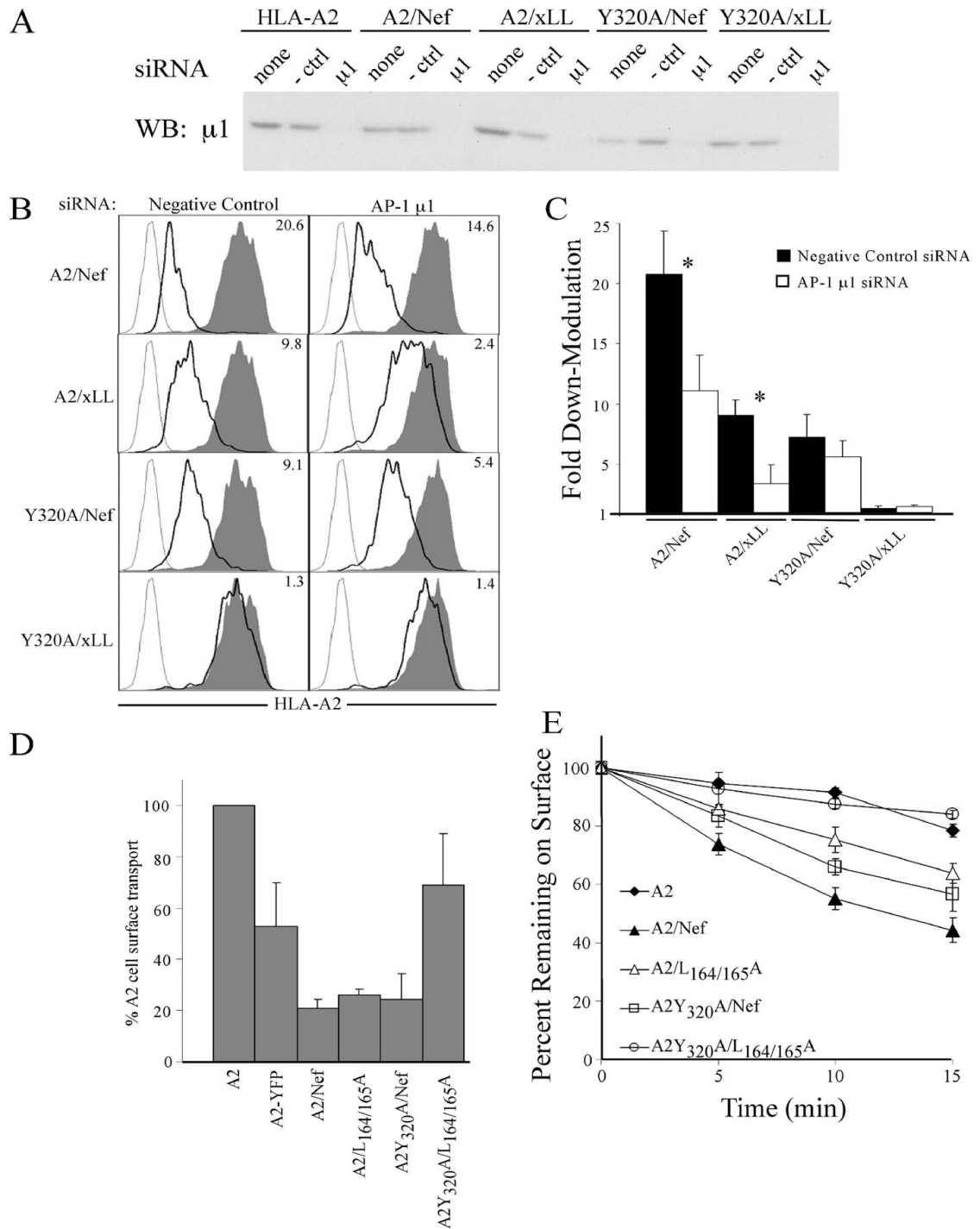


Figure 2.7. Trafficking signals within the A2/Nef fusion differ with regard to AP-1 dependence.

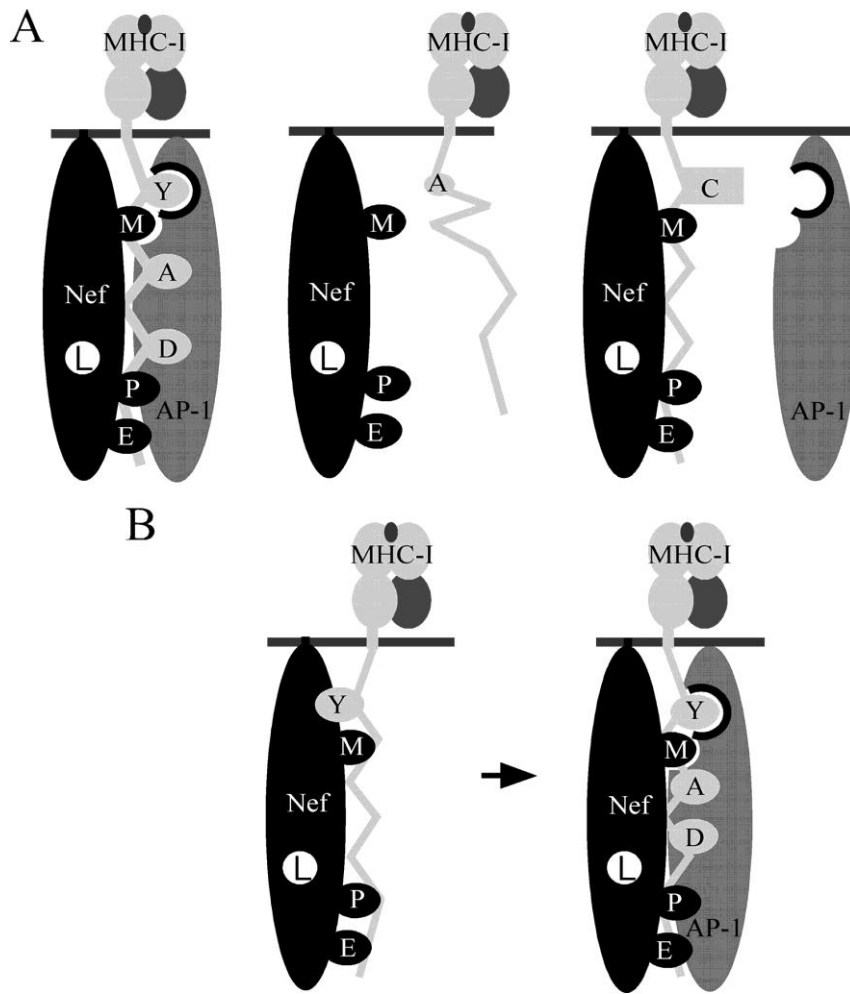


Figure 2.8. Proposed models of the Nef-MHC-I-AP-1 complex.

(A) Y₃₂₀ is necessary for maintaining the proper structure of the Nef binding site and for recruiting AP-1 via direct contact with the AP-1 tyrosine-binding pocket (highlighted in black). Mutating Y₃₂₀ to alanine (A) allosterically disrupts the Nef binding site, and AP-1 is no longer recruited. Cysteine (C) substitutions for Y₃₂₀ are tolerated for Nef binding, but this complex is unable to recruit AP-1 because there is no tyrosine available to interact with AP-1. Nef stabilizes the interaction between MHC-I and AP-1 through contacts with its methionine at position 20 (M), its polyproline helix (P) and its acidic domain (E). According to our model, when Nef is in the conformation needed for binding to MHC-I, its dileucine motif (L) is buried and unable to function as a trafficking signal. (B) Y₃₂₀ binds Nef and AP-1 sequentially. In this model Nef binds to MHC-I via Y₃₂₀ early in the secretory pathway (35), and subsequently, upon AP-1 binding in the *trans*-Golgi network, Y₃₂₀ interacts only with AP-1.

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CHAPTER III

HIV-1 Nef Targets MHC-I and CD4 for Degradation Via a Final Common β -COP-Dependent Pathway in T Cells

Abstract

To facilitate viral infection and spread, HIV-1 Nef disrupts the surface expression of the viral receptor (CD4) and molecules capable of presenting HIV antigens to the immune system (MHC-I). To accomplish this, Nef binds to the cytoplasmic tails of both molecules and then, by mechanisms that are not well understood, disrupts the trafficking of each molecule in different ways. Specifically, Nef promotes CD4 internalization after it has been transported to the cell surface, whereas Nef uses the clathrin adaptor, AP-1, to disrupt normal transport of MHC-I from the TGN to the cell surface. Despite these differences in initial intracellular trafficking, we demonstrate that MHC-I and CD4 are ultimately found in the same Rab7⁺ vesicles and are both targeted for degradation via the activity of the Nef-interacting protein, β -COP. Moreover, we demonstrate that Nef contains two separable β -COP binding sites. One site, an arginine (RXR) motif in the N-terminal α helical domain of Nef, is necessary for maximal MHC-I degradation. The second site, composed of a di-acidic motif located in the C-terminal loop domain of Nef, is needed for efficient CD4 degradation. The requirement for redundant motifs with distinct roles supports a model in which Nef exists in multiple conformational states that allow access to different motifs, depending upon which cellular target is bound by Nef.

Introduction

The HIV-1 accessory protein, Nef, affects the biology of the infected cell in several ways to achieve conditions optimal for viral replication and spread. Nef alters the intracellular trafficking of important immune molecules, such as class I and II major histocompatibility complex proteins (MHC-I and MHC-II), CD4, CD28, and DC-SIGN (1-5). Nef-dependent reduction of surface MHC-I protects HIV-infected primary T cells from recognition and killing by HIV-specific cytotoxic T lymphocytes (CTLs) *in vitro* (6). Moreover, disruption of MHC-I expression by HIV-1 and SIV Nef provides a selective advantage under immune pressure *in vitro* (7-10). CD4 downregulation by Nef is also essential for efficient viral spread. The rapid removal of CD4 prevents viral superinfection (11), and enables optimal viral particle production by eliminating detrimental CD4/HIV envelope interactions in the infected cell (12,13).

Mutagenesis of protein-protein interaction domains has revealed that Nef uses genetically separable mechanisms to affect MHC-I and CD4 transport. Specifically, disruption of MHC-I surface expression requires an N-terminal α helix, a polyproline repeat, and an acidic domain in Nef (14,15), while CD4 downregulation requires an intact dileucine motif, two diacidic motifs, and a hydrophobic pocket in Nef (15-18). Amino acids necessary for the myristoylation (19,20) and oligomerization (21) of Nef are required for the disruption of both MHC-I and CD4 surface expression.

Nef has the capacity to affect MHC-I transport at multiple subcellular locations; Nef blocks the export of newly-synthesized MHC-I from the secretory pathway and Nef expression results in a small increase in the rate of MHC-I internalization (22). To accomplish this, Nef directly binds to the cytoplasmic tail of MHC-I early in the secretory pathway (23-26). The Nef-MHC-I complex then actively recruits the clathrin adaptor protein complex AP-1, which targets MHC-I from the TGN to the endolysosomal network where it is ultimately degraded (25). Recruitment of AP-1 primarily requires a methionine at position 20 in the N-terminal α helical domain of Nef and a tyrosine residue in the cytoplasmic tail of MHC-I. Additionally, the acidic and polyproline domains of Nef have recently been shown to stabilize this interaction (27,28). The normal function of AP-1 is to target proteins into the endosomal pathway and then

recycle them back to the TGN. Thus, the AP-1 interaction with the Nef/MHC-I complex explains the targeting of MHC-I containing vesicles to the endosomal pathway and to the TGN. However, it does not explain accelerated degradation of MHC-I, hence other cellular factors may be involved (25).

The mechanism of Nef-induced CD4 internalization and degradation has been derived, in part, from correlating Nef function with the requirement for domains in the C-terminal flexible loop region of Nef that bind to cellular factors. The Nef dileucine motif (ExxxLL₁₆₅) is needed for CD4 internalization and it binds to adaptor protein complexes AP-1, AP-2, and AP-3 (16,29-36). In addition, a diacidic motif, which is also required, enhances the interaction of Nef with AP-2 (37). There is separate evidence that this diacidic motif may recruit the H subunit of the vacuolar ATPase (V1H) (38) to promote AP-2 recruitment (39). Because the normal role of AP-2 is to link cargo to clathrin and promote internalization, it makes sense that this molecule would be necessary and indeed, the involvement of AP-2 has now been confirmed using RNAi knockdown in a number of cell systems (40-42).

After CD4 is internalized, it is targeted to lysosomes for degradation. There is evidence that this step requires β -COP (18), a component of COP-1 coats implicated in endosomal trafficking as well as transport through the early secretory pathway (43-45). Specifically, there are defects in the Nef-dependent transport of CD4 into acidified vesicles at the non-permissive temperature in cells harboring a temperature sensitive ϵ -COP mutant (18). Nef directly interacts with β -COP (46), and a second diacidic motif in the C-terminal loop domain of Nef has been demonstrated to mediate this interaction (18,47), although, this result has not been reproducible by another group (48).

To more clearly understand the mechanism of altered MHC-I and CD4 trafficking observed in Nef-expressing cells, we directly compared these two processes in T cells that expressed Nef. We confirmed that Nef primarily affected MHC-I and CD4 at different subcellular locations and we demonstrated that the cytoplasmic tails of the respective molecules dictated which pathway was utilized. Despite the differences in initial trafficking, we found that HLA-A2 and CD4 co-localized in a discrete subset of vesicular structures. Upon further inspection, we determined that these structures also contained markers of late endosomes (Rab7) and to a lesser extent, the lysosomal marker,

LAMP-1. Electron microscopy (EM) revealed that CD4 and HLA-A2 were found within MVBs of Nef-expressing T cells. HLA-A2 (but not CD4) was also found in tubulovesicular structures adjacent to the Golgi. In Nef expressing cells, reduction of β -COP expression reduced the targeting of HLA-A2 from the TGN to LAMP-1⁺ compartments and stabilized CD4 expression within endosomal compartments. Finally, we identified two separate domains within Nef that were necessary for these activities and for β -COP binding. These data support a model in which both MHC-I and CD4 are ultimately targeted to the lysosomes in Nef expressing cells by a final common pathway.

Materials and Methods

Cell lines. CEM T cells stably expressing HA-tagged HLA-A2 (CEM HA-HLA-A2) have already been described (25). Cell lines stably expressing YFP-tagged Rab7 or HA-HLA-A2/CD4 were made by transducing cells with murine retroviral constructs (MSCV YFP-Rab7 or MSCV HA-A2/CD4) as previously described (22), followed by culture in selective media.

DNA constructs. MSCV YFP-Rab7 was constructed by cloning a filled-in a Kpn I-Xho I fragment from pEYFP-Rab7 (49) into MSCV puro (50). MSCV HA-A2/CD4 was constructed using PCR mutagenesis. The first round PCR produced two products: the first utilized 5' primer (primer 1) 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and 3' primer (primer 2) 5'-CTCTGCTTGGCGCCTTCGGTGCCACATCACAGCAGCGACCAC-3' with MSCV HA-HLA-A2 as the template (25). The second utilized 5' primer (primer 3) 5'-GTGGTCGCTGCTGTGATGTGGCACCGAAGGCGCCAAGCAGAG-3' and 3' primer (primer 4) 5'-CCTCGAGTCAAATGGGGCTACATGTCTTCTGAAATCGGTGAGGGCACTGG-3' using CD4 as the template. The second round utilized primers 1 and 4 from the previous PCR reactions plus 1 µl of each purified first round PCR reactions as template. The resulting product was digested with BamHI and XhoI and ligated into MSCV 2.2 (50) digested with BglII and XhoI.

MSCV A2/Nef has been described (26). MSCV HA-A2/CD4/Nef was constructed using a PCR mutagenesis approach. The first round PCR produced two products: the first utilized 5' primer (primer 1) 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and 3' primer (primer 2) 5'-CCACTTGCCACCCATACTAGTAATGGGGCTACATGT-3' with MSCV HA-A2/CD4 as the template. The second utilized 5' primer (primer 3) 5'-ACATGTAGCCCCATTACTATGATGGGTGGCAAGTGG-3' and 3' primer (primer 4) 5'-GCGAATTCTCAGCAGTTCTTGAAGTACTC-3' with NL4-3 Nef open reading frame as template. The second round utilized primers 1 and 4 from the previous PCR reactions plus 1 µl of each purified first round PCR reactions as template. The resulting product was digested with BamHI and EcoRI and ligated into MSCV IRES GFP (51) digested with BglII and EcoRI.

Nef mutants were made by using the PCR mutagenesis approach described previously (27). The mutagenesis primers were as follows: R_{17/19}A 5'-TGGCCTACTGT-AGCGGAAGCAATGAGACGAGCT-3' and EE₁₅₄₋₁₅₅AA 5'-GTTGAGCCAGATAAG-GTAGCAGCGGCCAATAAAGGAGAGA-3'. Each primer, plus its reverse complement were utilized together with additional 5' and 3' primers to generate the mutated product. Wild type NL4-3 Nef [MSCV A2/Nef IRES GFP (25) was used as a template for the PCR reaction, except for the double mutant, R_{17/19}A/EE₁₅₄₋₁₅₅AA, in which the MSCV R_{17/19}A Nef IRES GFP was used as the template. Each mutated PCR product was digested and cloned into MSCV IRES GFP (51) as described previously (27).

The FG12 shRNA lentiviral vectors were constructed as previously described (52). Briefly, complementary primers were annealed together and ligated into vector pRNAi (53) digested with BglII and HindIII. The sequences of the primers were as follows (the target sequence is underlined): shNC (an siRNA directed at GFP, with several base changes (25))- sense 5'-GATCCCCGCTCACACTGAAGTTAATCTTCA-AGAGAGATTA ACTTCAGTGTGAGCTTTTTGGAAA-3', antisense 5'-AGCTTTTC-CAAAAAGCTCACACTGAAGTTAATCTCTCTTGAAGATTA ACTTCAGTGTGAGC GGG-3', sh β -COP- sense 5'-GATCCCCTGAGAAGGATGCAAGTTGCTTCAAGAG-AGCAACTTGCATCCTTCTCATT TTTGGAAA-3', antisense 5'-AGCTTTTCCAAA-ATGAGAAGGATGCAAGTTGCTCTCTTGAAGCAACTTGCATCCTTCTCAGGG-3'; sh μ 1A- (a mixture of two lentiviruses was used) (1) sense 5'-GATCCCCTGAGGTG-TTCTTGGACGTCTTCAAGAGAGACGTCCAAGAACACCTCATT TTTGGAAA-3', antisense 5'-AGCTTTTCCAAAATGAGGTGTTCTTGGACGTCTCTCTTGAAGAC-GTCCAAGAACACCTCAGGG-3', (2) sense 5'- GATCCCCCGACAAGGTCCTCTTT-GACTTCAAGAGAGTCAAAGAGGACCTTGTCGTTTTTGGAAA-3', and antisense 5'- AGCTTTTCCAAAACGACAAGGTCCTTTGACTCTCTTGAAGTCAAAGA-GGACCTTGTCGGGG-3'. The pRNAi constructs were digested with XbaI and XhoI to remove the promoter and shRNA sequence. The resulting fragment was ligated into FG12 (52), digested with XbaI and XhoI.

Virus preparation and transductions. Adenovirus was prepared by the University of Michigan Gene Vector Core facility. Adenoviral and HIV (HXB-EP (6)) transductions of T cells (25) or 373 MG astrocytoma cells (54) have been described

previously. Murine retroviral vector (MSCV) expressing Nef was prepared as described previously (25), except that in some cases the retroviral vector supernatants were concentrated by spinning at 14000 RPM for four hours at 4°C. The viral pellet was then resuspended in media to yield a twenty-fold concentrated stock. Lentiviruses expressing shRNA were generated using an approach similar to that already described (52). Briefly, 293 cells were transfected with the FG12 constructs described above plus pRRE (55), pRSV-Rev (55) and pHCMV-G (56) using Lipofectamine 2000 (Invitrogen). Supernatants from the transfected cells were collected and used to transduce CEM T cells using a spin-transduction protocol.

Flow cytometry and internalization assays. Intact cells were stained for flow cytometry analysis as previously described (24). Briefly, HLA-A2 was detected with BB7.2 (57) that had been purified as previously described (22). Endogenous CD4 was detected using RPA-T4 from Serotec. The secondary antibody was goat anti-mouse-phycoerythrin (Bio- Source, 1:250). For experiments using the GFP-expressing FG12 lentivirus for shRNA expression, the GFP-positive cells were gated to identify the subset of transduced cells (generally >90% of cells). Endocytosis assays were performed as previously described with minor modification (22). Briefly, cells were washed once with Endocytosis Buffer [D-PBS, 10 mM HEPES, 10 µg/ml BSA (NEB)], then stained with primary antibody (described above) for 20 minutes on ice. After washing, the cells were resuspended in RPMI supplemented with 10% fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, penicillin and streptomycin (R10) (pre-warmed to 37°C) and replicate aliquots were removed and placed on ice for each time point. Cells were then washed and stained with goat anti-mouse-phycoerythrin (BioSource, 1:250) and the samples were analyzed using a FACScan flow cytometer (Becton Dickinson). Flow cytometry data was processed using FlowJo v4.4.3 software (Treestar Corp.). The mean fluorescence at time zero was set to 100%, and this value was used to calculate the relative surface staining at each subsequent time point.

Cell surface transport assay. CEM cells transduced with adenoviral vectors as previously described (22) were first incubated in pre-label media [RPMI – Cys –Met (Specialty Media, Inc.)+10% dialyzed FBS (Invitrogen)] for 15 minutes at 37°C. Pulse labeling was performed in pre-label media with 150–200 µCi/ml Pro-mix-L [³⁵S] (>1000

Ci/mmol; Amersham Pharmacia) for 30 minutes at 37°C. The cells were then chased in R10 media for 15 minutes at 37°C, followed by two washes with D-PBS. To label the protein that reached the cell surface, the cells were resuspended in D-PBS containing 0.5 mg/ml EZ-Link sulfo-NHS-LC-Biotin (Pierce), and incubated at 37°C for 1 hour. Surface biotinylation was quenched by washing the cells in D-PBS+25 mM Lysine (Fisher).

For Figure 3.1D, immunoprecipitation of proteins from cell lysates was performed as previously described (25), except that one-third of the total lysate was used for the HLA-A2 immunoprecipitation while two-thirds of the material was used to recover CD4. For immunoprecipitations of ³⁵S labeled proteins, 5 µg of BB7.2 and 2.5 µg RPTA4 (BD Pharmingen) were used for HLA-A2 and CD4 respectively. In Figure 3.1E and 3.5D, the total cell lysate was immunoprecipitated with anti-HA ascites (HA.11, Covance).

For Figures 3.1D, 3.1E and 3.5D, recovered proteins were released from the beads by boiling in 100 µl of 10% SDS. One third was analyzed directly by SDS-PAGE (Total). The remaining two thirds was brought to a total volume of 1 ml in RIPA Buffer (25), and 40 µl of avidin-agarose (Calbiochem) was added to recover biotinylated proteins. After 2 hours at 4°C, the beads were washed three times with 1 ml RIPA buffer and proteins were separated by SDS-PAGE (Surface).

Immunofluorescence microscopy. Adeno-transduced CEM cells were adhered to glass slides, fixed, permeabilized, and stained for indirect immunofluorescence as previously described (25). Bafilomycin treatment was performed as described previously (25). The following antibodies were utilized to localize proteins via microscopy: Figure 3.2, and Figures 3.3 and 3.9; anti-CD4 (S3.5, Caltag Laboratories) and anti-HLA-A2 (BB7.2); Figure 3.5; anti-giantin (Covance); Figure 3.8; anti-CD4 antibody (S3.5, Caltag Laboratories), anti-LAMP-1 (H4A3, BD Pharmingen) and anti-HLA-A2 (BB7.2). Secondary antibodies were obtained from Molecular Probes and were used at a dilution of 1:250: Giantin, Alexa Fluor 546 goat anti-rabbit; CD4, Alexa Fluor 546 goat anti-mouse IgG2a; LAMP-1, Alexa Fluor 546 goat anti-mouse IgG1; BB7.2 (Figures 3.2, 5D and S4), Alexa Fluor 647 goat anti-mouse IgG2b; BB7.2 (Figure 3.3), Alexa Fluor 488 goat anti-mouse IgG2b. See Table 3.1 for a summary of antibodies used to gather data for Table 3.2.

For the microscopy based internalization assay in Figure 3.8A, CEM T cells were allowed to adhere to glass slides, and placed on ice. The cells were washed once with wash buffer (D-PBS, 10 $\mu\text{g/ml}$ BSA (NEB) and 2% goat serum), incubated with anti-CD4 antibody (S3.5, Caltag Laboratories, IF, 1:25) for 20 minutes, washed once with wash buffer, incubated with Alexa fluor 546 goat anti-mouse IgG2a (Molecular Probes, 1:250) for 20 minutes and washed once with wash buffer. The zero time point was fixed with 2% paraformaldehyde, while the remaining time points incubated at 37°C for the indicated time. The cells were then fixed with 2% paraformaldehyde. Images were collected using a Zeiss LSM 510 confocal microscope and processed using Adobe Photoshop software. Three-dimensional projections of cells were generated from Z-stacks using Zeiss LSM Image Examiner software. Otherwise, single Z sections through the center of the cell were displayed.

Electron microscopy. Electron microscopy with CEM cells transduced with adenovirus was performed by the Harvard Medical School (HMS) Electron Microscopy Facility. Frozen samples were sectioned at -120°C, the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of parafilm. All antibodies and protein A gold were diluted in 1% BSA. The diluted antibody solution was centrifuged 1 minute at 14,000 rpm prior to labeling to avoid possible aggregates. Grids were floated on drops of 1% BSA for 10 minutes to block for unspecific labeling, transferred to 5 μl drops of primary antibody and incubated for 30 minutes. The grids were then washed in 4 μl drops of PBS for a total of 15 minutes, transferred to 5 μl drops of Protein-A gold for 20 minutes, washed in 4 μl drops of PBS for 15 minutes and 6 μl drops of double distilled water. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops (diameter slightly larger than the grid) and the excess liquid was removed by streaking on a filter paper (Whatman #1), leaving a thin coat of methyl cellulose (bluish interference color when dry). The grids were examined in a Tecnai G² Spirit BioTWIN transmission electron microscope and images were recorded with an AMT 2k CCD camera.

Western blot analyses and immunoprecipitations. For the western blot analysis in Figures 3.4, 3.5A, 3.6C, 3.7, 3.11D, and 3.13A cells were lysed in PBS 0.3% CHAPS, 0.1% SDS pH 8, 1 mM PMSF, normalized for total protein and separated by SDS-PAGE. Endo H (NEB) digestion was performed according to the manufacturer's protocol. Staining of the western blot was performed using anti-Nef (AG11, (58)) and anti- β -COP (M3A5 (59)), which were purified as previously described (22). Additional antibodies used were HA (Covance) and μ 1 (RY/1 [75]). The secondary antibody for anti-Nef, β -COP, and HA was HRP-rat anti-mouse IgG1 (Zymed) and for anti- μ 1 was HRP-goat antirabbit (Zymed).

For Figure 3.10B, the IP-western experiment was performed as previously published (26). Briefly, parental CEM T cells were spin-transduced with murine retroviral supernatant expressing either empty vector, A2/Nef or A2/CD4/Nef. At 72 hours post transduction, the cells were incubated in 20 mM NH₄Cl for 4 hours. The cells were then treated with DTBP (Pierce) for 40 minutes, quenched per the manufacturer's protocol, and lysed in PBS with 0.3% Chaps and 0.1% SDS. The lysate was precleared and immunoprecipitated for HLA-A2 with BB7.2 chemically crosslinked protein A/G beads (Calbiochem) (25). The immunoprecipitates were washed in TBS with 0.3% CHAPS and 0.1% SDS. A more stringent IP protocol was used in Figures 3.10A, 3.11C, 3.12E, and 3.14. For these experiments, CEM cells were transduced with control, wild type Nef, or mutant Nef expressing adenovirus (Figure 3.10A and 3.11C) or concentrated MSCV (Figures 3.12E and 3.14). At 48 hours post-transduction, the cells were incubated in 20 mM NH₄Cl for 16 hours. The cells were not crosslinked and were lysed in digitonin lysis buffer (1% digitonin (Wako), 100 mM NaCl, 50 mM Tris pH 7.0, 1 mM CaCl₂, and 1 mM MgCl₂). After pre-clear, the lysates were immunoprecipitated with either BB7.2 (Figures 3.10A and 3.14) or M3A5 (Figures 3.11C and 3.12E) crosslinked to beads. The immunoprecipitates were eluted and analyzed by western blot as described previously (26).

Pulse-chase analysis of protein degradation. A total of 30 million CEM T cells transduced with wild type or mutant Nef using concentrated MSCV as described above were pulse labeled for 30 minutes with [³⁵S]-methionine and cysteine. Half of the cells were collected as the zero time point and stored at -20°C. The remaining cells were then

chased for 12 hours in RPMI, collected and stored at -20°C. Lysates were generated in lysis buffer (PBS 0.3% CHAPS, 0.1% SDS pH 8, 1 mM PMSF) and precleared overnight. They were immunoprecipitated for two hours with an anti-HLA-A2 antibody (BB7.2) and washed once in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). The immunoprecipitates were then eluted by boiling in 10% SDS, reprecipitated with an antibody against HA (HA.11, Covance), and washed two times in RIPA buffer. The final immunoprecipitates were then separated by SDS-PAGE, the gel was dried down and analyzed using a phosphorimager.

Molecule Detected	Primary Ab	Secondary Ab
HLA-A2	BB7.2 (20 µg/ml)	GAM-IgG2b AlexaFluor 647 (1:250, Molecular Probes)
CD4	RPAT4 (1:50, BD Pharmingen)	GAM-IgG1 AlexaFluor 546 (1:250, Molecular Probes)
YFP-Rab7	NA	NA
HLA-A2	BB7.2 (20 µg/ml)	GAM-IgG2b AlexaFluor 488 (1:250, Molecular Probes)
CD4	α-CD4-FITC (1:25, Caltag)	NA
γ-adaptin	Clone 88 (1:25, BD Pharmingen)	GAM-IgG1 AlexaFluor 546 (1:250, Molecular Probes)
EEA1	Clone 14 (1:200, BD Pharmingen)	GAM-IgG1 AlexaFluor 546 (1:250, Molecular Probes)
Lamp1	H4A3 (1:500, BD Pharmingen)	GAM-IgG1 AlexaFluor 546 (1:250, Molecular Probes)

Table 3.1. Combinations of antibodies used for immunofluorescence staining for experiments summarized in Table 3.2.

Results

The cytoplasmic tail dictates the pathway utilized by Nef to eliminate MHC-I and CD4 surface expression. It is known that Nef binds to the cytoplasmic tails of both CD4 and MHC-I, but that it affects them differently. To better understand the similarities and differences governing these two pathways, we examined the trafficking of CD4, HLA-A2 and a chimeric molecule in which the wild type HLA-A2 cytoplasmic tail was substituted with the CD4 cytoplasmic tail (HA-A2/CD4). A flow cytometric analysis of steady state surface expression revealed that Nef dramatically reduced steady state surface expression of all three molecules (Figure 3.1A). Consistent with prior studies, we found that CD4 was rapidly internalized from the cell surface in Nef expressing T cells, whereas wild type HLA-A2 was not (Figure 3.1B). Substitution of the CD4 tail for the HLA-A2 cytoplasmic tail was sufficient to confer this phenotype (Figure 3.1C). Conversely, prior studies have shown that Nef disrupts cell surface expression of MHC-I by blocking the transport of newly synthesized MHC-I from the TGN to the cell surface (22,23). As shown in Figure 3.1D, Nef inhibited HLA-A2 forward transport by approximately 75%, whereas CD4 was unaffected at Nef levels that had a clear effect on HLA-A2 transport. Slight effects on CD4 could be observed at higher Nef levels (Figure 3.1D, lane 8). The substitution of the HLA-A2 cytoplasmic tail with the CD4 tail reduced the ability of Nef to disrupt forward trafficking (Figure 3.1E). Thus, sequences in the cytoplasmic tails of CD4 and HLA-A2 determine how Nef disrupts their trafficking.

CD4 and a subset of HLA-A2 proteins are found in late endosomes and lysosomes of Nef-expressing T cells. To better understand the similarities and differences between MHC-I and CD4 trafficking in Nef-expressing cells, we compared the steady-state distribution of these molecules in T cells using confocal microscopy (Figure 3.2A). We found that Nef expression caused the bulk of MHC-I to cluster in the perinuclear region where, in agreement with many other studies (14,30,54), it co-localized with markers of the TGN. Interestingly, we also identified a subset of HLA-A2 that co-localized with CD4 in vesicular structures (Figure 3.2A; arrows show example vesicles). To further identify these structures, we simultaneously stained for HLA-A2, CD4, and organelle markers using 3-color confocal microscopy (summarized in Table

3.2). Our results indicated that CD4 was mainly found in discrete vesicular structures, which also contained HLA-A2 (91.9% of the CD4⁺ vesicles co-localized with HLA-A2, Table 3.2) and markers of late endosomes and lysosomes. Overall, the best marker for structures containing both HLA-A2 and CD4 was Rab7 (94%, of CD4⁺ vesicles co-localized with Rab 7, Table 3.2 and Figure 3.2A, arrowheads mark example vesicles). CD4 and HLA-A2 were also found to co-localize with markers of lysosomes, such as LAMP-1. However, the vesicles with the most intense LAMP-1 staining did not contain either HLA-A2 or CD4, possibly because of degradation. Consistent with this, the co-localization of HLA-A2 and CD4 was dramatically increased when the cells were treated with bafilomycin, which inhibits degradation in acidic compartments (Figure 3.3). Thus, the normal steady-state co-localization of HLA-A2 and CD4 in Nef expressing cells was limited because degradation prevented accumulation in this compartment.

Colocalization of HLA-A2 and CD4 in MVBs. To further discern these structures, we also examined them using electron microscopy (EM). In agreement with the confocal data, our EM analysis revealed that compared with control cells in which both HLA-A2 and CD4 were found on the cell surface (Figure 3.2B, panel 1), in Nef-expressing T cells, the majority of CD4 was found in MVBs, co-localizing with HLA-A2 (Figure 3.2B, panel 2). In addition, we also noted substantial HLA-A2, but not CD4, accumulating in tubulovesicular structures adjacent to Golgi stacks (Figure 3.2B, panel 3). In separate experiments these structures were also found to contain AP-1 (Figure 3.2C). Based on these studies, it appears that the majority of HLA-A2 resides in tubulovesicular structures in the region of the TGN with AP-1, whereas at any given time, a small subset can be found in the endosomal compartment with CD4.

Required cellular co-factors. To further elucidate the similarities and differences between these pathways, we examined the role of known Nef-interacting proteins implicated in intracellular trafficking. AP-1 is a heterotetrameric adaptor protein involved in protein sorting from the TGN and it has been previously demonstrated to interact with MHC-I molecules in Nef expressing HIV-infected primary T cells and to direct MHC-I into the endolysosomal pathway (25). Nef is also known to interact with β -COP (46), a component of COP-1 vesicles also involved in endosomal trafficking (43-

45). Indeed, expression of wild type COP-1 components is needed for targeting CD4 into acidic vesicles in Nef-expressing cells (18).

To compare and contrast the requirement for these factors in Nef-dependent CD4 and HLA-A2 trafficking, we knocked down their expression using lentiviral vectors expressing short hairpin RNAs (shRNAs) (52). All of these studies were performed in T cells and new cell lines were generated for each experiment to eliminate the possibility that long term growth in culture would select for cells that had compensated for the defect. Using this system, we obtained good knock down of the μ 1 subunit of AP-1 and β -COP (Figure 3.5A–C). (A small apparent effect of sh β -COP on μ 1 levels observable in Figure 3.5A was not significant when adjusted for protein loading in the experiment shown here or in replicate experiments (Figure 3.5B). We also did not observe any effect of another siRNA directed against a different target site in β -COP on μ 1 expression (Figure 3.4).)

The effect of knocking down β -COP expression on the structural integrity of the Golgi. Because β -COP is known to be important for intra-Golgi and ER-to-Golgi trafficking, we asked whether the Golgi structure or MHC-I trafficking were drastically affected by reduced β -COP expression. We found that there was only a small reduction in the normal transport of MHC-I to the cell surface (35% reduction, Figure 3.5D). In addition, cells lacking β -COP generally maintained overall Golgi structure as assessed by the intracellular localization of giantin, a transmembrane protein normally residing in the cis and medial Golgi (60) (Figure 3.5E). In contrast, brefeldin A, an inhibitor of an ARF1 GEF necessary for β -COP activity obliterated the normal Golgi staining (Figure 3.5E, panel 9). The relatively mild phenotype of this knock-down compared to the drastic effects of brefeldin A, suggests that brefeldin A has effects other than just disrupting COP-1 coats by blocking ARF1 activity.

Having established that knocking down β -COP allowed relatively normal forward trafficking of HLA-A2, we proceeded to assess the effect of knocking down β -COP or AP-1 in Nef-expressing cells. Consistent with previous publications (25), we found that knocking down the ubiquitously expressed form of AP-1 (AP- μ 1A (61)) largely reversed the effect of Nef on HLA-A2 ($p < 10^{-4}$), but had a smaller and less significant effect ($p < 0.02$) on CD4 surface expression (Figure 3.6A and 4B). Surprisingly, we also

observed that knocking down β -COP expression inhibited MHC-I downmodulation by Nef and had a small but statistically significant effect on CD4 downmodulation ($p < 10^{-3}$; Figure 3.6A and 4B). The small effect of β -COP on CD4 surface expression indicated that β -COP was not necessary for CD4 internalization and downmodulation from the cell surface. However, further studies were needed to determine whether β -COP was required to degrade the CD4 after it was internalized.

A role for β -COP in promoting degradation of Nef cellular targets. Prior studies had determined that expression of β -COP was necessary for acidification of CD4-containing vesicles and thus it was hypothesized that β -COP was needed to target vesicles containing internalized CD4 for lysosomal degradation. Therefore, we asked whether the role of β -COP in MHC-I trafficking was also to promote MHC-I degradation. To examine this, we utilized an assay we had developed, which measures the loss of mature, endo H-resistant HA-tagged HLA-A2 in Nef expressing cells by western blot analysis. This assay system is based on previous data demonstrating Nef-dependent degradation of the mature form of MHC-I in a manner that is reversible by inhibitors of lysosomal degradation (25). As shown in Figure 3.6C, under normal, steady state conditions, most of the HLA-A2 is resistant to endo H digestion, indicating that it has matured through the Golgi apparatus (Figure 3.6C, lane 2). However, when Nef was expressed, we observed a dramatic reduction in total MHC-I and a decrease in the ratio of endo H resistant to sensitive protein (Figure 3.6C compare lanes 2 and 18, see also Figure 3.7). Consistent with a role for AP-1, we observed that AP-1 μ 1 A shRNA largely reversed this effect of Nef (Figure 3.6C, compare lanes 18 and 20. See also Figure 3.6D for quantification). To detect degradation of molecules containing a CD4 tail, we used HA-A2/CD4 (Figure 3.1) and found that Nef expression accelerated the degradation of endo H resistant forms of this molecule (Figure 3.6C, compare lanes 6 and 22). However, we found that there was no effect of reduced AP-1 μ 1 A expression on Nef-dependent degradation of molecules containing the CD4 tail (Figure 3.6C, compare lanes 22 and 24. See also Figure 3.6D for quantification).

When β -COP expression was reduced, we observed a small increase in the amount of immature, endo H-sensitive protein (Figure 3.6C, compare lanes 10 and 12), consistent with the 35% reduction in export of MHC-I to the cell surface shown in Figure

3.5D. However, we also noted that reduction in β -COP expression reduced the Nef-dependent degradation of the mature, endo H resistant form of these molecules (Figure 3.6C, compare lanes 26 and 28. See also Figure 3.6D for quantification) implicating β -COP in this pathway. We were also able to confirm the model that β -COP is involved in Nef-dependent CD4 degradation as treating cells with β -COP shRNA reduced the degradation of the A2/CD4 chimeric molecule (Figure 3.6C, compare lanes 30 and 32. See also Figure 3.6D for quantification).

β -COP is required for targeting internalized CD4 for degradation in Nef-expressing T cells. We next directly examined the effect of reducing β -COP expression on Nef-dependent trafficking by confocal microscopy. For these experiments, cells were infected with HIV or were transduced with Nef-expressing adenoviral vectors and then the fate of internalized CD4 was assessed by confocal microscopy. Using this assay system, we observed fairly rapid internalization of CD4 in Nef-expressing cells, followed by loss of CD4 staining by 30 minutes (Figure 3.8A, compare control cells in row 1 to Nef-expressing cells in row 3). However, in T cells expressing β -COP shRNA, there was a three-to-four fold increase in the number of CD4-containing vesicles, consistent with a role for β -COP in promoting maturation of these vesicles into degradative compartments (Figure 3.8A, compare control treated Nef-expressing cells in row 3 to sh β -COP-expressing cells in row 4). Reduction of β -COP expression yielded similar results whether Nef was introduced using HIV infection or via adenoviral vectors (Figure 3.8B and 3.8C).

β -COP is required for targeting MHC-I to LAMP-1⁺ compartments in Nef-expressing T cells. Confocal analysis of MHC-I intracellular localization revealed that expression of β -COP shRNA in control cells increased the intracellular accumulation of MHC-I, consistent with the slowing of export we observed in cells deficient in β -COP (Figure 3.8D, compare rows 1 and 2). Infection with Nef-expressing HIV resulted in the loss of cell surface MHC-I and an increase in intracellular MHC-I, some of which colocalized with LAMP-1 (Figure 3.8D, compare rows 1 and 3). Under these conditions, reduction of β -COP expression reduced the degree of colocalization with LAMP-1 (Figure 3.8D, compare rows 3 and 4). To enhance our ability to observe trafficking of MHC-I into LAMP-1⁺ compartments, we treated the cells with bafilomycin, which

inhibits the vacuolar ATPase and thus acidification and degradation within lysosomal compartments. As previously reported (25), bafilomycin treatment enhanced our ability to detect MHC-I in LAMP-1⁺ compartments in Nef-expressing T cells (Figure 3.8D, compare rows 3 and 7). The expression of β -COP shRNA decreased LAMP-1 colocalization with MHC-I, consistent with a role for β -COP in targeting MHC-I for degradation in lysosomal compartments in Nef expressing T cells (Figure 3.8D, compare rows 7 and 8). Similar results were observed whether Nef was introduced using HIV or adenoviral vectors (Figure 3.8E and 3.8F).

We also examined co-localization of HLA-A2 and CD4 in cells that expressed β -COP shRNA. We observed that reduction of β -COP expression resulted in increased staining of both proteins, and did not disrupt their co-localization (Figure 3.9). Thus, β -COP was not necessary for targeting these proteins into a common endosomal pathway, but rather was needed for their subsequent targeting into a degradative pathway.

The cytoplasmic tail of MHC-I is necessary for AP-1 binding in Nef-expressing T cells. To further explore the molecular mechanism for the similarities and differences in MHC-I and CD4 trafficking in Nef-expressing T cells, we asked whether these molecules differed as to how well they bound Nef or cellular factors. As expected, we found that HIV Nef bound to both the HLA-A2 and the CD4 tail (Figure 3.10A, right panel). However, AP-1 only co-precipitated with molecules containing the HLA-A2 cytoplasmic tail (Figure 3.10A, right panel). The chimeric molecule with the CD4 cytoplasmic tail did not bind AP-1 in Nef-expressing T cells (Figure 3.10A, right panel). In these experiments, we noted that the expression level of A2/CD4 was lower than for wild type HLA-A2, which could explain this difference. Therefore, we confirmed these data using a fusion protein containing either HLA-A2 or A2/CD4 directly fused to full length HIV-Nef protein. In previously published experiments it was shown that the HLA-A2/Nef fusion protein co-precipitated AP-1 in a manner that depended on sequences both in Nef and in the HLA-A2 cytoplasmic tail (25). Here we show again that the HLA-A2 cytoplasmic tail was necessary for this interaction and, moreover, that the CD4 tail could not substitute for it (Figure 3.10B, right panel).

Evidence that formation of a Nef- β -COP complex is an essential step necessary for MHC-I degradation. The Nef- β -COP interaction is well-described in the

literature (46) and there is evidence that β -COP interacts with a diacidic motif (E_{154/155}) within the Nef C-terminal loop (18). However, this region of Nef has never been implicated in MHC-I trafficking. To provide further evidence that β -COP is needed to promote MHC-I degradation, we sought to identify a region of Nef that is needed both for MHC-I degradation as well as β -COP binding. We therefore examined a panel of mutations (M₂₀A, V₁₀E Δ 17–26 and E_{62–65}Q) that are specifically defective at disrupting MHC-I trafficking (14,15,26,62). We also examined a Nef mutant, D₁₂₃G, that is defective at both CD4 and MHC-I downmodulation (21). The relative activity of these Nef mutants in MHC-I and CD4 downmodulation is shown in Figure 3.11A and quantified in Figure 3.11B.

We then examined the relative ability of each of these mutant molecules to co-precipitate with β -COP. As shown in Figure 3.11C, we found that the V₁₀E Δ 17–26-Nef, which is defective at MHC-I downmodulation, was also defective at binding to β -COP (compare lanes 3 and 5). Interestingly, this deletion mutant is also defective at interacting with AP-1 (25). However, the β -COP binding site was separable from the AP-1 interaction site because M₂₀, which is located within the deleted region, is needed for AP-1 interaction (25,27), but was not necessary for β -COP binding to Nef (Figure 3.11C, compare lanes 3 and 4). Mutation of the Nef dimerization motif [D₁₂₃G, (21)], which disrupts a number of Nef functions, including MHC-I and CD4 downmodulation, also reduced binding to β -COP (Figure 3.11C, compare lanes 3 and 7). Finally, mutation of the Nef acidic domain (E_{62–65}Q), which disrupts binding to MHC-I (26), AP-1 (27,28) and PACS-1 (63), did not affect binding to β -COP (Figure 3.11, compare lanes 3 and 6).

As expected, we found that V₁₀E Δ 17–26 Nef, which was defective at β -COP binding, was also defective at inducing the degradation of the endo H resistant form of HLA-A2 (Figure 3.11D, upper panel, compare lanes 3 and 4 with lanes 5 and 6). In contrast, V₁₀E Δ 17–26 Nef was not defective at A2/CD4 degradation based on western blot analysis (Figure 3.11D, lower panel, compare lanes 3 and 4 with lanes 5 and 6). These data suggested that there may be another interaction domain that recruits β -COP to the Nef- CD4 complex to promote CD4 degradation. This would be consistent with the faint band observable in the V10E Δ 17–26-Nef mutant immunoprecipitation (Figure

3.11C, lane 5, longer exposure) and prior publications demonstrating that mutation of E_{154/155} also affected β -COP binding (47). Thus, there may be two independent binding sites for β -COP within Nef, each of which governs the degradation of a different cellular factor.

To further define the β -COP binding site, and to determine whether there were indeed two β -COP binding sites, we constructed additional Nef mutants. We focused on the arginine residues (R₁₇ER₁₉MR₂₁R₂₂) within the Nef deletion Δ 17–26) because previous studies had indicated that arginine rich regions could form β -COP-binding sites (64). Flow cytometric analysis of MHC-I levels on cells expressing these mutants revealed that the R_{17/19} pair was necessary for maximal MHC-I downmodulation (Figure 3.12A and B). In contrast, mutation of R_{21/22} did not significantly affect MHC-I downmodulation. An assessment of Nef-induced degradation by pulse chase analysis of HA-HLA-A2, revealed that mutating this motif also inhibited Nef-dependent degradation (Figure 3.12C, compare lanes 5 and 7, quantified in Figure 3.12D). Additionally, mutation of R_{17/19} reduced, but did not eliminate binding of β -COP to Nef in a manner similar to the effect of the Δ 17–26 Nef mutation (Figure 3.12E, compare lanes 3 and 4).

We next examined the diacidic motif (E_{154/155}) previously implicated in β -COP binding. As shown in Figure 3.12A and B, mutation of this motif did not disrupt MHC-I downmodulation, in fact downmodulation was somewhat enhanced. Additionally, we found that mutation of this motif did not reduce MHC-I degradation (Figure 3.12C, compare lanes 5 and 11, see also quantification in 3.12D). However, in agreement with prior results, we observed a partial defect in β -COP binding with this mutant (Figure 3.12E, compare lanes 3 and 6, (18,47). However, this defect was less reproducible (observed in two out of four experiments) than that observed with disruption of R_{17/19} (consistently observed in five out of five experiments), suggesting that binding to R_{17/19} can mask the defect observed with mutation of E_{154/155} under certain conditions. To provide additional data supporting the possibility that both sites contributed to β -COP binding, we constructed a double mutant, R_{17/19} A and E_{154/155}A (R/E). As shown in Figure 3.12E, lane 5, binding of R/E to β -COP was further reduced relative to binding of Nef proteins containing single mutations in each motif, strongly implicating both

motifs in β -COP binding. The phenotype of the double mutant was highly reproducible in 5 out of 5 experiments.

Interestingly, the R/E double mutant was not more defective than R_{17/19}A at downmodulating MHC-I (Figures 3.12A and B) or at promoting MHC-I degradation (Figure 3.12C, compare lanes 7 and 9, quantified in 3.12D), indicating that Nef did not utilize the E_{154/155} binding site to recruit β -COP for MHC-I degradation. Conversely, we confirmed prior reports that the E_{154/155}A mutant was defective at CD4 degradation (Figure 3.13A, compare lanes 3 and 6) and determined moreover that there was no significant effect of mutating R_{17/19} on CD4 degradation, either alone or in combination with E_{154/155}A (Figure 3.13A, compare lanes 3 and 4). It is also worth noting that, in contrast to what was observed with HLA-A2, we did not observe a clear correlation between the relative CD4 surface expression and the relative level of total cellular CD4 (compare Figure 3.12B and 3.13B), indicating that there was a complex relationship between total cellular CD4 and the fraction expressed on the cell surface.

Because the R_{17/19} motif is directly adjacent to M₂₀, which is necessary for AP-1 recruitment (25,27), we also examined whether these mutations, which affect β -COP binding, also disrupted AP-1 co-precipitation. To accomplish this, we used our standard AP-1 recruitment assay in which proteins co-precipitating with MHC-I HLA-A2 were detected by western blot analysis. As shown in Figure 3.14, mutation of R_{17,19} (and E_{154/155}) decreased AP-1 binding only slightly. Thus, the defects in MHC-I downmodulation and degradation noted with mutation of R_{17,19} resulted primarily from defects in β -COP binding.

Discussion

Expression of HIV Nef in infected cells protects them from lysis by CTLs and this activity of Nef is due to downmodulation of MHC-I surface expression. The Nef protein also prevents superinfection and promotes viral spread by removing the viral receptor, CD4 from the cell surface (for review see (65)). We provide evidence that sequences in the cytoplasmic tail of these molecules are important for determining whether Nef disrupts their trafficking from the cell surface or at the TGN. These data, that swapping cytoplasmic domains switches the initial pathways taken by HLA-A2 and CD4 in the presence of Nef, may seem somewhat obvious. Nef is always the same and thus one might conclude that this information has to be contained in the modulated protein. However, it was also possible that the ectodomain affected Nef responsiveness by binding to other transmembrane proteins or by altering intracellular trafficking. This was certainly a possibility for MHC-I for which it is clear that the efficiency of peptide loading can affect trafficking and we have found that trafficking rates affect responsiveness to Nef and AP-1 binding (23).

Prior studies have demonstrated that Nef initially binds to hypo-phosphorylated forms of the MHC-I cytoplasmic tail early in the secretory compartment (23), but binding does not affect normal transit through the Golgi apparatus and into the TGN (25). The Nef-MHC-I complex then recruits the AP-1 heterotetrameric clathrin adaptor protein using a binding site that is created when Nef binds the MHC-I cytoplasmic tail. This binding site requires a methionine from the N-terminal α helix of Nef and a tyrosine residue in the MHC-I cytoplasmic tail (25). Additionally, there is evidence that this complex is stabilized by the acidic and polyproline domains of Nef (27,28). Formation of this complex results in the re-direction of MHC-I trafficking in such a way that it is targeted to lysosomes for degradation (25). However, cellular proteins that normally bind AP-1 are not degraded, but rather recycled to the TGN (Figure 3.13C). Here we present new evidence that Nef utilizes β -COP to promote trafficking to degradative compartments (Figure 3.13C). Knocking down expression of β -COP inhibited the degradation of MHC-I and it did so by blocking the transport of MHC-I from intracellular vesicles to LAMP-1⁺ compartments. We also provide results here that confirm β -COP is

necessary for degradation of CD4 in lysosomal compartments. Thus, we propose that AP-1 and AP-2 deliver MHC-I and CD4 respectively to endosomal compartments where β -COP displaces AP-1 and AP-2 to target MHC-I and CD4 for lysosomal degradation (Figure 3.13C).

As described above, we found that knocking down β -COP with shRNA resulted in stabilization of internalized CD4, however the effect on CD4 surface expression was small, but still significant. In contrast, there was a greater effect of β -COP knockdown on HLA-A2 surface expression. This might suggest that the role of β -COP in the modulation of these targets was different, rather than the same. However, this apparent paradox can be explained by our model shown in Figure 3.13C. As indicated, differences in response to β -COP knockdown can be explained by differences in the intracellular pathways of these proteins before they interact with β -COP. MHC-I is engaged in an AP-1-dependent endosome-to-TGN loop, and MHC-I could “leak” out to the cell surface from the TGN in the absence of β -COP, whereas CD4 may be unable to return to the cell surface from its endosomal compartment. Consistent with this, we also noted a lack of correlation between degradation and surface expression of CD4 (but not MHC-I) when Nef mutants that were defective in β -COP binding were examined. These data indicate that there is a complex relationship between total cellular CD4 and the fraction that is present on the cell surface and thus intracellular pools need to be directly examined to assess degradation rather than relying on surface expression as an indicator of the efficiency of this process.

It is also noteworthy that shRNA knockdown of β -COP did not fully reverse Nef-dependent MHC-I and CD4 degradation. This may have resulted from incomplete knockdown of β -COP. However, we also observed a similar phenotype with Nef mutants defective at β -COP binding. Failure to fully reverse degradation may be secondary to a default degradative pathway that exists for all proteins delivered to endosomal pathways. Alternatively, there may be other ways Nef targets these proteins to lysosomes, which have yet to be identified.

Our studies indicate that there are at least three domains needed for Nef to interact efficiently with β -COP. One of these domains (D₁₂₃), is required for dimerization of Nef and is needed to affect a variety of Nef functions (21). Another region lies within the N-

terminal α helical domain of Nef that is specifically required for disruption of MHC-I trafficking and for interactions with AP-1 (25). This binding site for β -COP is distinct from that used by AP-1, because recruitment of β -COP does not require Nef's acidic domain or Nef M₂₀, whereas AP-1 does (25,27). The fact that these Nef mutants bind β -COP, but are still defective at MHC-downmodulation (64) makes sense, because these mutants are also unable to bind the MHC-I cytoplasmic tail (26).

Additional mutants, which focused on the highly conserved stretch of arginines in the N-terminal alpha helical domain of Nef (R₁₇XRMRR₂₂), revealed that the regions involved in AP-1 and β -COP binding were very closely apposed. However, we determined that mutation of R_{17/19} affected primarily β -COP binding, with only a minimal effect on AP-1 interaction. Thus, these two Nef-interacting proteins have distinct and separable amino acid requirements for binding.

The identification of a β -COP binding domain within a region of Nef that is also required for Nef to accelerate MHC-I degradation confirms the requirement for β -COP in this pathway. In addition, the residual binding of β -COP to these Nef mutants provided suggestive data that another binding site for β -COP existed. Indeed, we were able to confirm prior evidence that a diacidic motif within the C-terminal loop of Nef also promoted an interaction with β -COP and that mutation of this motif reduced CD4 degradation (47). Finally, we demonstrated that mutation of both the RXR and the diacidic motifs resulted in the greatest defect in β -COP binding. The double mutant did not however result in a greater defect in either MHC-I or CD4 degradation, indicating the role of each motif is distinct and not additive. The discovery of two distinct β -COP binding motifs helps explain why some groups could not confirm the role of the diacidic motif in β -COP binding (48) as both motifs need to be mutated to reliably eliminate an interaction between β -COP and Nef.

There is precedent for such redundancy. For example, there are two AP-1 binding sites within Nef; a dileucine motif within the C-terminal flexible loop (16,31-33) as well as a second site that forms upon binding of Nef to the MHC-I cytoplasmic tail. Despite the presence of two AP-1 signals, only one is active in the context of the natural Nef-MHC-I complex (25,27). The dileucine motif in the C-terminal flexible loop can become activated to affect MHC-I transport, but only when Nef is artificially fused to the MHC-I

cytoplasmic tail (27). This result indicates there is no inherent inability of this signal to affect MHC-I traffic but rather that something else, such as the structure of the natural complex, causes the dileucine motif to be inactive (27). The dileucine motif at position 164 is located close to the diacidic motif at position 154 that binds β -COP to promote CD4 degradation. The fact that both of these motifs are inactive when Nef is bound to MHC-I, suggests that much of the C-terminal flexible loop region of Nef is inaccessible under these conditions. Thus, Nef behaves as though it assumes different structural forms in different contexts to differentially expose distinct trafficking signals.

We also present evidence that knock down of β -COP yielded a distinct phenotype from BFA treatment. As described above, BFA is a chemical inhibitor of ARF1, that is known to trigger the reversible collapse of the cis-medial Golgi compartments to the ER (66-68) by inhibiting an ARF-specific guanine nucleotide-exchange protein (ARF-GEF) (69,70). Because ARF1 activity is necessary for recruitment of β -COP to membranes (71), it was possible that the dramatic effects of BFA resulted from the inability for β -COP to function normally. However, our results demonstrating that knockdown of β -COP had no effect on overall Golgi structure indicate that the dramatic effects of BFA are not due solely to disruption of β -COP function in the Golgi.

Given the important role of β -COP in the Golgi, it is surprising that β -COP bound to Nef does not also affect transport of MHC-I through the ER/Golgi. It is possible that our inability to detect an effect of Nef on early transport of MHC-I (25) may be a result of the cell type chosen for these studies. T cells, which are an important natural target of HIV, normally traffic MHC-I through the early secretory pathway slowly (23) and thus it might be difficult to further reduce the trafficking speed through an interaction with β -COP. Interestingly, another group has reported a reduced ER-Golgi exit rate for MHC-I in Nef-expressing HeLa cells (72), which normally transport MHC-I more rapidly than T cells (23). We have made similar observations in astrocytoma cells expressing higher levels of Nef than typically needed to observe MHC-I downmodulation (Roeth and Collins, unpublished observations). Further studies will be needed to determine whether this effect of Nef plays a role in more physiologically relevant cell systems and whether this effect of Nef might be dependent on β -COP expression.

A recent report indicates that the effect of Nef on internalization of MHC-I, which is only minimally apparent in our system, occurs via a PI3-kinase dependent pathway (73). This publication reported that CEM cells, which were used in our study, have less PTEN (a phosphatase that inhibits PI3-kinase) than another T cell line used in their study (H9). This deficiency might make it relatively more difficult for us to detect an effect of chemical PI3-kinase inhibitors, but would not affect our ability to detect a PI3-kinase-dependent trafficking pathway. In fact, one would expect the opposite, that the PI3-kinase-dependent pathway would be more active in our system. However, we have found that Nef has a relatively small effect on internalization of MHC-I, and mainly affects MHC-I protein export and degradation. These data have been corroborated in HIV-infected primary T cells (22,26) which were also found to much lower levels of PTEN than H9 cells did (73).

From a teleological perspective, it makes sense that Nef would have evolved to target early forms of MHC-I, which harbor antigens derived from the newly synthesized viral proteins. Older forms of MHC-I already on the cell surface would be bound to normal cellular antigens and would in fact be protective as they would inhibit killing by natural killer cells that are stimulated to lyse cells with abnormally low MHC-I expression. On the other hand, it makes sense that Nef, an early viral protein, would have evolved to target surface CD4 to rapidly and efficiently remove CD4 in order to prepare the cell for rapid release of viral particles and to render the cell resistant to re-infection. Meanwhile, a late protein, Vpu, is expressed in infected cells and specifically targets the newly synthesized CD4 for degradation, preventing any additional CD4 from reaching the cell surface (74).

In sum, we have found that the HIV Nef protein commandeers the cellular trafficking machinery efficiently by utilizing their natural activities for abnormal purposes. The fact that these pathways may end in a final common step raises the important possibility that inhibitors might be developed that could block multiple Nef functions.

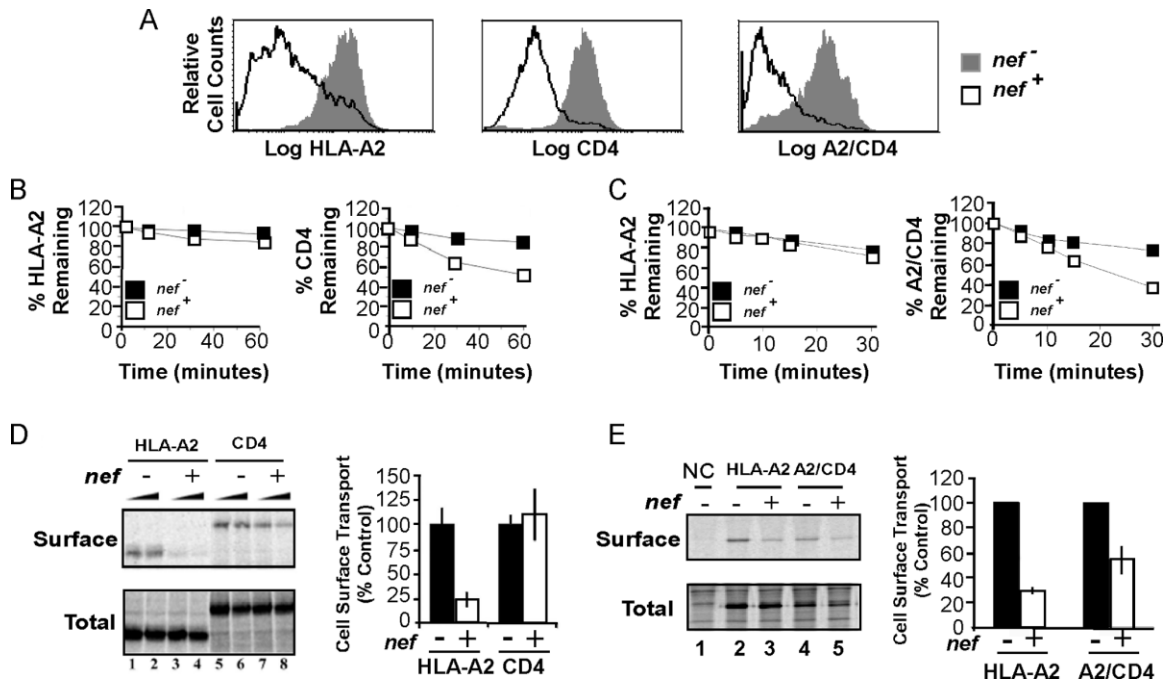


Figure 3.1. The cytoplasmic tail domain of MHC-I and CD4 determines the mechanism by which Nef affects trafficking.

(A) Reduction of surface expression of HLA-A2, CD4 and A2/CD4 as measured by flow cytometry. CEM HA-HLA-A2 and CEM HA-A2/CD4 cells were transduced with a control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺) and stained for surface HLA-A2 and CD4. The histograms shaded gray represent cells treated with control adenovirus, and the solid black line indicates cells treated with adeno-Nef. (B-C) Measurements of surface stability. CEM HA-HLA-A2 and HA-A2/CD4 cells were treated with adenovirus as in part A and the internalization of endogenous CD4 (B) or A2/CD4 (C) was compared to the internalization of HLA-A2. The filled squares represent control (*nef*⁻) cells, and the open squares represent adeno-Nef (*nef*⁺) cells. Quantitation of part B was compiled from four independent experiments performed in duplicate. Part C is representative of two experiments performed in triplicate. (D-E) HLA-A2 is inefficiently transported to the cell surface in T cells expressing Nef. CEM HA-HLA-A2 and CEM HA-A2/CD4 cells were transduced as in part A. Metabolic labeling with continuous surface biotinylation was performed in the presence of a cell-impermeable biotinylation reagent [NHS-biotin, (Pierce)] to label cell surface proteins. The cells were lysed and immunoprecipitated first with an antibody against HLA-A2 or CD4 (part D) or anti-HA (part E), then 2/3 was re-precipitated with avidin beads to selectively precipitate the HLA-A2 on the cell surface. Normalized surface MHC-I was calculated as follows: ((surface MHC-I /total MHC-I×2)×100). Quantitation for parts D and E represents the mean±standard deviation for four and three independent experiments respectively. For part D, quantitation is derived from data for the lower of the two Nef levels shown.

Marker	Organelle	Number of colocalizing vesicles	Total Number of CD4 positive vesicles	% CD4 Colocalization
HLA-A2	N/A	306	333	91.9
γ -adaptin	TGN/Endosomes	29	78	37.2
EEA1	Early Endosomes	48	125	38.4
YFP-Rab7	Late Endosomes	49	52	94.2
Lamp1	Lysosomes	92 ¹	130	70.8

¹The majority of CD4 co-localized with weakly Lamp1-positive structures.

Table 3.2. Analysis of CD4⁺ structures in Nef-expressing T cells.

CEM HLA-A2 cells were transduced with adeno-Nef and analyzed by three-color confocal microscopy as described in Materials and Methods. Discrete CD4⁺ structures were identified and scored for co-localization with HLA-A2 or the indicated organelle marker protein. Data from at least two independent experiments were combined for each protein analyzed.

Figure 3.2. MHC-I and CD4 co-localize in a subset of vesicles in Nef-expressing T cells. (A) Three-way co-localization of HLA-A2, CD4 and Rab7. CEM HLA-A2 cells stably expressing YFP-Rab7 were transduced with adeno-Nef. HLA-A2 (red), CD4 (green), and YFP-Rab7 (blue) were simultaneously detected using three-color confocal microscopy. The top row shows the x - y projection of the cell, while the bottom row displays the x - z projection. Ten sequential optical sections were compiled to generate a projection of each cell about the x - z plane. Scale bar = 5 microns. (B) Immunogold labeling of HLA-A2 and CD4. Representative electron micrographs of CEM HA-HLA-A2 cells treated with control adeno (column 1) or adeno-Nef (columns 2 and 3). Thawed cryosections of cells were labeled with anti-HA (HLA-A2) and anti-CD4 antibodies followed by 15 and 10 nm protein A-gold respectively. (C) Immunogold labeling of HA-HLA-A2 and γ -adaptin in Nef-expressing CEM T cells. Thawed cryosections of cells were labeled with anti-HA (HLA-A2) and anti- γ -adaptin antibodies followed by 15 and 10 nm protein A-gold, respectively.

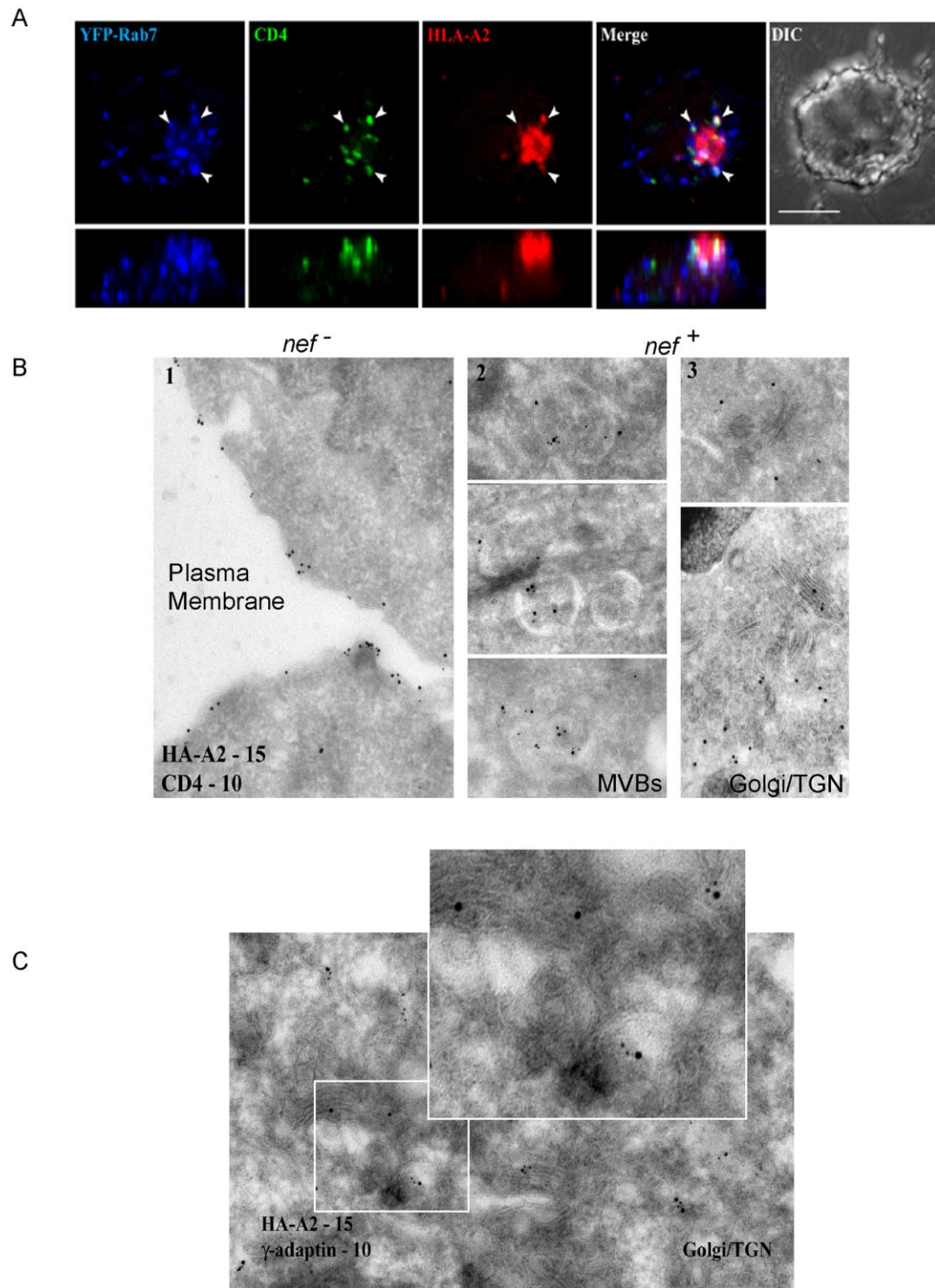


Figure 3.2. MHC-I and CD4 co-localize in a subset of vesicles in Nef-expressing T cells.

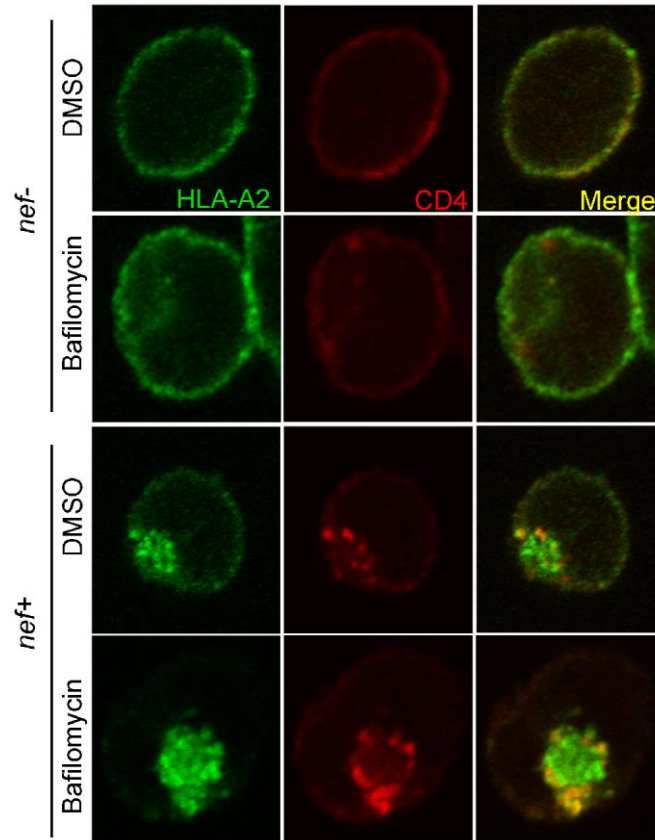


Figure 3.3. Bafilomycin treatment increases MHC-I and CD4 co-localization in Nef-expressing cells.

CEM HA-HLA-A2 cells were transduced with a control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺) as described in Materials and Methods. At 72 hours later, the cells were treated with bafilomycin or solvent control (DMSO) and stained with antibodies directed against HLA-A2 and CD4 as described in Materials and Methods. Images were taken with a Zeiss confocal microscope and processed with LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown.

Figure 3.4. Knockdown of β -COP does not affect HLA-A2 transport to the cell surface or disrupt the Golgi apparatus. (A) Analysis of protein expression in β -COP and μ 1 knockdown cells. CEM HA-HLA-A2 cells were transduced with a lentivirus expressing both GFP and a control shRNA (shNC) or an shRNA targeting either β -COP (sh β -COP) or μ 1 (sh μ 1). At 72 hours later, they were transduced with adeno-Nef or control adenovirus. Three days later they were harvested, and western blot analysis was used to assess protein levels of β -COP, μ 1 and Nef. (B,C) Quantification of μ 1 and β -COP expression in shRNA treated cells. The amount of either μ 1 (B) or β -COP (C) was quantified using Adobe Photoshop software. The average percent remaining \pm standard deviation for four experiments (B) and three experiments (C) is shown. To adjust for protein loading in part B, the nonspecific background band directly below μ 1 (shown in part A) was used to normalize protein loading. (D) Knockdown of β -COP does not affect HLA-A2 transport to the cell surface. CEM HA-HLA-A2 cells were transduced with lentivirus expressing either shNC or sh β -COP as in part A. Cell surface transport was assessed using a metabolic labeling assay with biotinylation as described in Figure 3.1D. (E) Knockdown of β -COP does not disrupt the Golgi apparatus. CEM HA-HLA-A2 cells were transduced with lentivirus expressing the indicated shRNA and GFP as in part A and treated with brefeldin A (BFA) at 50 μ M or DMSO for 30 minutes. The integrity of the Golgi apparatus was assessed by immunofluorescence staining for giantin and analyzed by confocal microscopy. Images were taken using a Zeiss confocal microscope and analyzed with LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown. The results shown for parts D and E are representative of three independent experiments.

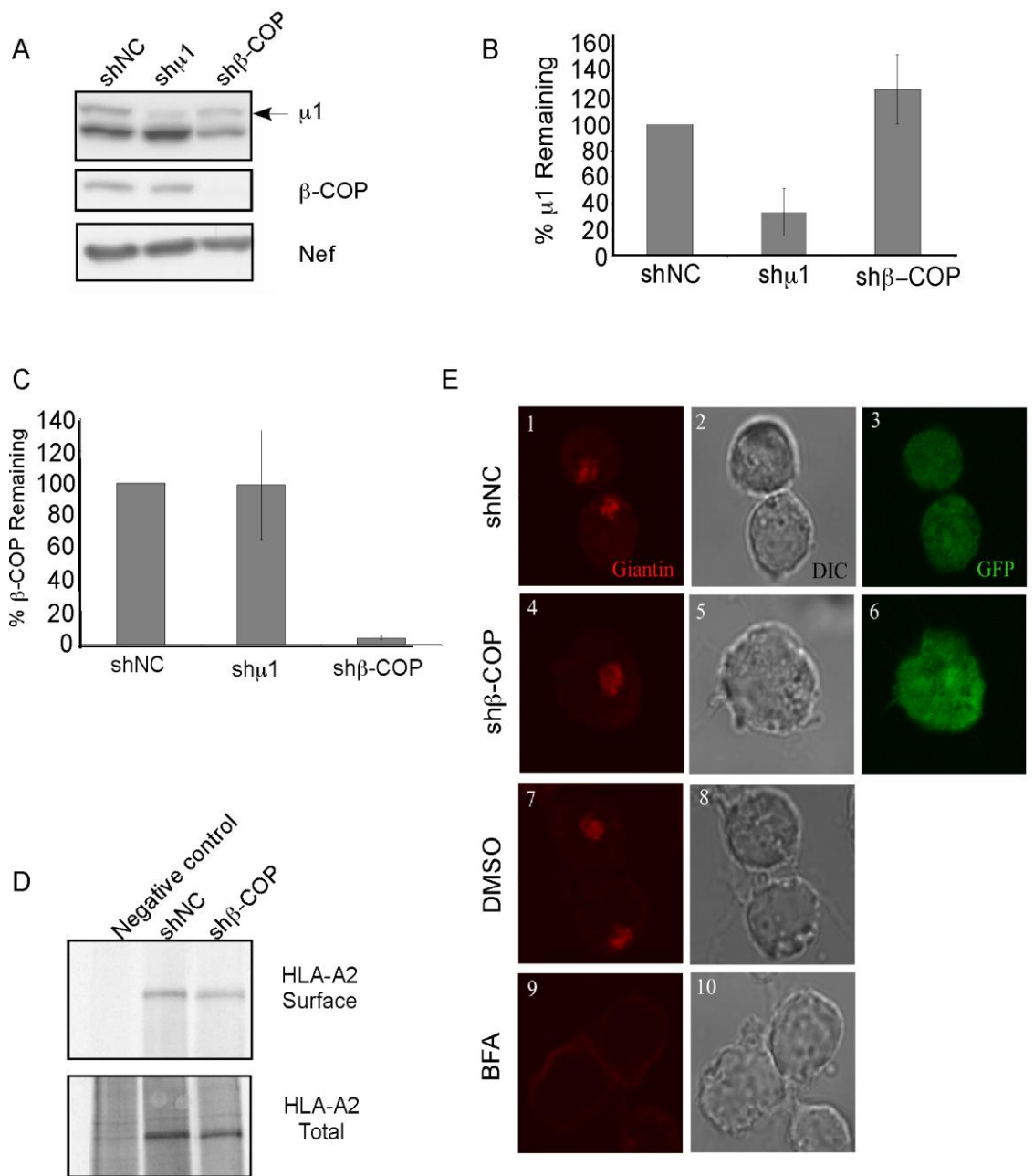


Figure 3.4. Knockdown of β -COP does not affect HLA-A2 transport to the cell surface or disrupt the Golgi apparatus.

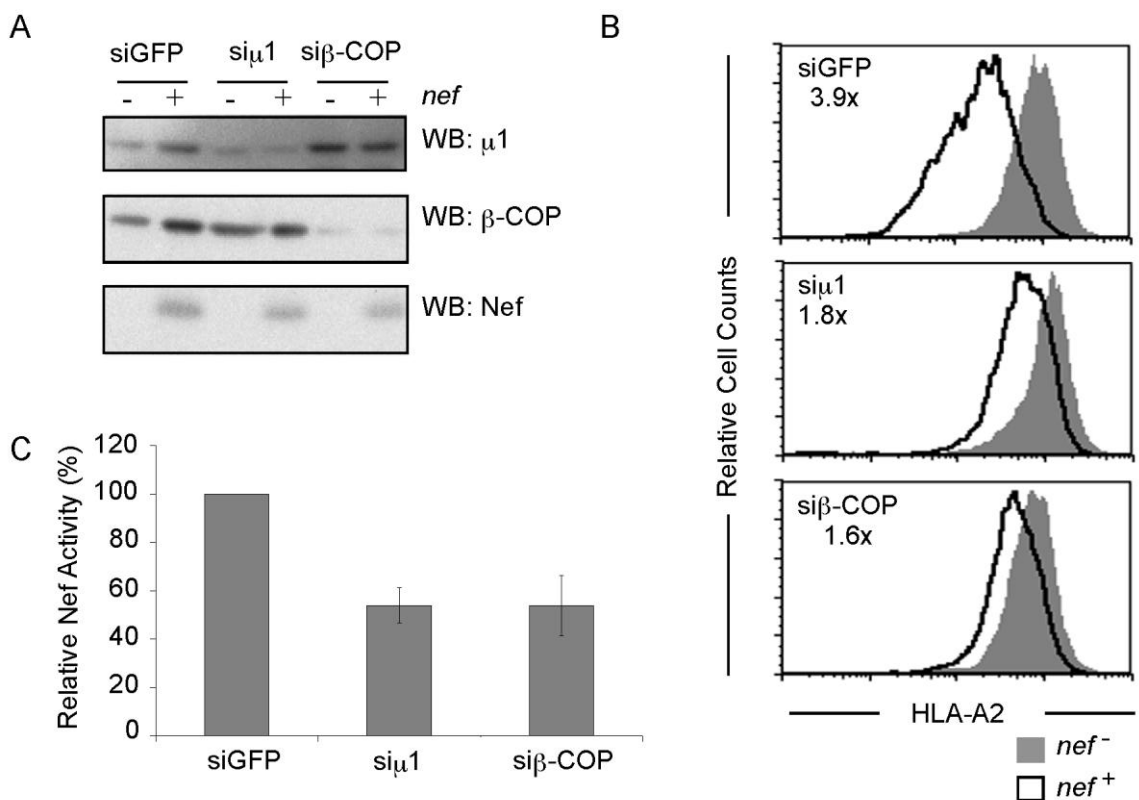


Figure 3.5. A second siRNA directed at β -COP disrupts Nef-dependent MHC-I trafficking.

(A) Western blot analysis of protein expression in 373 mg astrocytoma cells transfected with the indicated siRNA. Previously published protocols (25) were used to transfect 373 mg astrocytoma cells with control siRNA (siGFP (25)) an siRNA targeting β -COP (si β -COP, sense 5'-GGAGAUGUAAAGUCAAGA-3', antisense 5'-UCUUUGACUUUAC-AUCUCC-3', Ambion) or an siRNA targeting the AP-1 μ subunit (si μ 1 (25)). The data is representative of three experiments. (B) β -COP and μ 1 are required for Nef to efficiently reduce cell surface expression of HLA-A2. HLA-A2 cell surface expression on astrocytoma cells from (A) was assessed by flow cytometry as described in Materials and Methods. The fold downmodulation of HLA-A2 (mean fluorescence intensity of control/mean fluorescence intensity of Nef-expressing cells) for each condition is shown in the upper left corner. (C) Quantitation of HLA-A2 fold downmodulation in Nef expressing cells treated with siRNA. The mean fold downmodulation \pm standard deviation from three experiments is shown.

Figure 3.6. Nef requires β -COP to reduce HLA-A2 cell surface expression and accelerate HLA-A2 degradation. (A) β -COP and μ 1 are required for Nef to reduce cell surface expression of HLA-A2. CEM HA-HLA-A2 cells were transduced with a lentivirus expressing GFP and a control (shNC), β -COP (sh β -COP) or μ 1 (sh μ 1) shRNAs and with control adenovirus (*nef*⁻) or adeno-Nef (*nef*⁺). Cell surface expression of HLA-A2 or CD4 in the GFP-positive cells was assessed by flow cytometry. The gray shaded histogram represents control adenovirus (*nef*⁻) treated cells and the solid black line represents adeno-Nef (*nef*⁺) treated cells. (B) Quantitation of HLA-A2 and CD4 downmodulation in Nef expressing cells transduced with shRNA. The median fold downmodulation (median fluorescence of control/median fluorescence of Nef-expressing cells) \pm standard deviation derived from five (HLA-A2) and four (CD4) independent experiments. A *p*value was calculated using a two tailed t-test and significant differences were indicated with asterisks (**p*<0.02, ****p*<10⁻³, *****p*<10⁻⁴). (C) Knockdown of β -COP stabilizes intracellular levels of HLA-A2 and A2/CD4 in Nef expressing cells. CEM HA-HLA-A2 and CEM HA-A2/CD4 were treated as in part A. Lysates from these cells were generated and treated with endoglycosidase H (endo H). Protein levels of HLA-A2 and A2/CD4 were assessed by western blot using an anti-HA antibody. Endo H-resistant bands are marked with an R and endo H-sensitive bands are marked with an S. The results shown are representative of three independent experiments for HLA-A2 and two independent experiments for A2/CD4. (D) Quantification of endo H-resistant protein. Adobe Photoshop software was used to quantify each band for the Nef-expressing samples. The percentage of endo H-resistant protein in each condition was calculated as follows: [resistant band/(resistant band+sensitive band)] \times 100. The fold stabilization was then calculated as: (% endo H-resistant in experimental sample)/[% endo H-resistant in control (shNC)]. The data shown is the mean of two experiments \pm standard deviation.

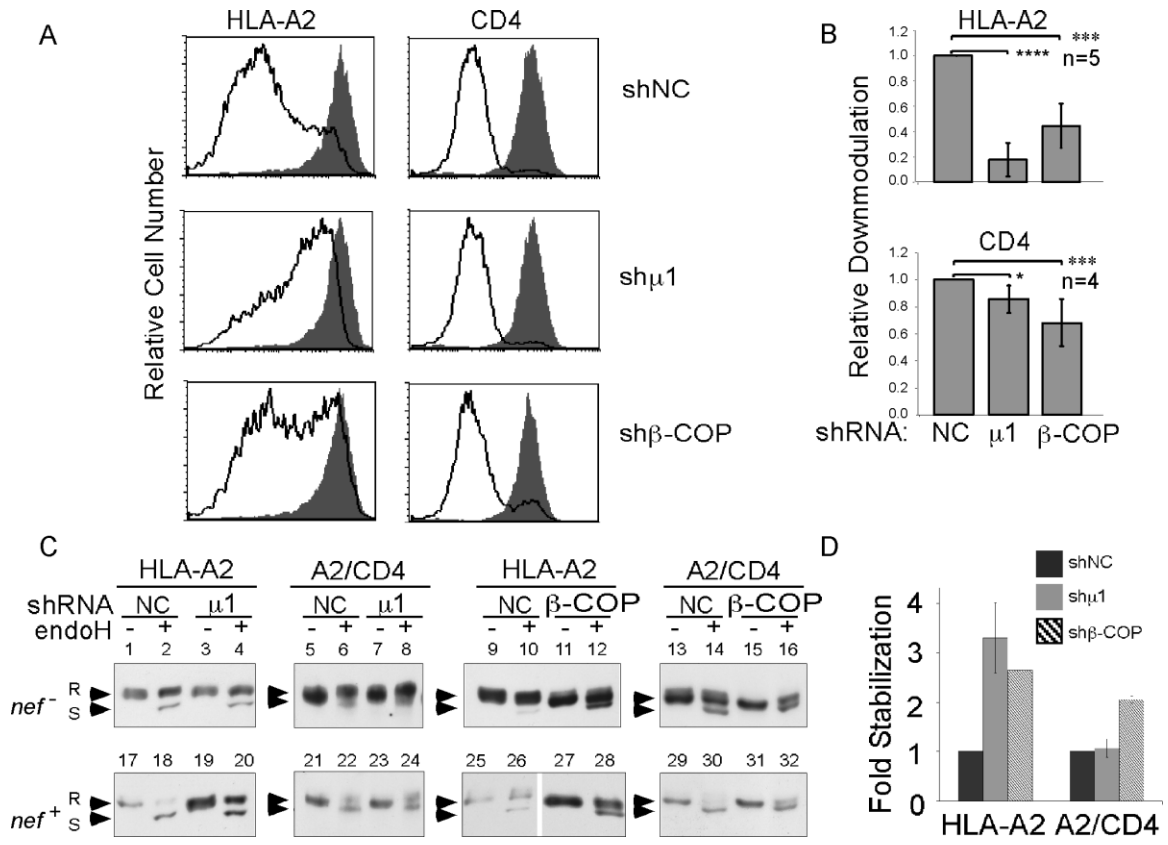


Figure 3.6. Nef requires β -COP to reduce HLA-A2 cell surface expression and accelerate HLA-A2 degradation.

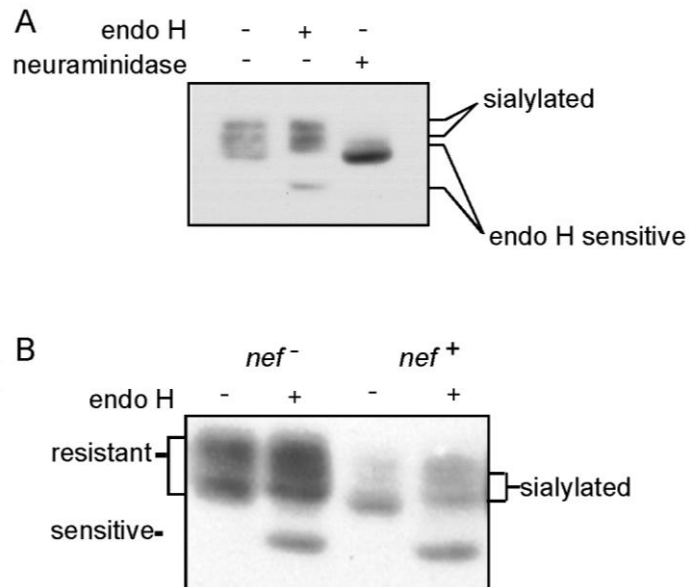


Figure 3.7. Characterization of HA-HLA-A2 protein forms using western blot analysis.

(A) CEM T cells expressing HA-HLA-A2 were lysed and treated with either Endo H or neuraminidase. The samples were then analyzed via Western blot. (B) CEM T cells expressing HA-HLA-A2 and Nef or a control adenoviral vector were lysed, normalized for total protein, digested with endo H, and probed for HA-HLA-A2 by Western blotting with an anti-HA antibody.

Figure 3.8. Nef requires β -COP to target HLA-A2 and CD4 for degradation. (A) Knockdown of β -COP stabilizes CD4⁺ vesicles in Nef expressing cells. CEM HA-HLA-A2 cells transduced with a lentivirus expressing GFP and either control shRNA (shNC) or shRNA targeting β -COP (sh β -COP) were transduced with control adenovirus (nef⁻) or adeno-Nef (nef⁺). The cells were incubated with CD4 antibody on ice and then shifted to 37°C for internalization for the indicated times. Images were taken with a Zeiss confocal microscope and processed using LSM Image Browser and Adobe Photoshop software. Single Z-sections are shown. (B) Quantitation of CD4⁺ vesicles is shown for 15 GFP⁺, nef⁺ cells treated with shNC and 17 GFP⁺, nef⁺ cells treated with sh β -COP. The mean \pm standard deviation is shown. (C) Quantitation is shown for 5 GFP⁺, nef⁺ cells treated with shNC and 5 GFP⁺, nef⁺ cells treated with sh β -COP. The mean \pm standard deviation is shown. (D) CEM HA-HLA-A2 cells were transduced with a lentivirus expressing either GFP and control (shNC) or β -COP (sh β -COP) shRNA, infected with HIV, treated with bafilomycin or DMSO and stained for HLA-A2 and LAMP-1 as previously described (25). Images were taken with a Zeiss confocal microscope and processed as in part A. Single Z-sections are shown. (E) Relative co-localization of HLA-A2 with LAMP-1 in 10 GFP⁺, adeno-Nef-expressing T cells treated with shNC and 15 GFP⁺, adeno-Nef-expressing T cells treated with sh β -COP. (F) Relative co-localization of HLA-A2 with LAMP-1 in 6 GFP⁺, HIV-nef⁺ infected T cells treated with shNC and, 6 GFP⁺, HIV-nef⁻ infected T cells treated with sh β -COP. Quantitation of microscopy data was performed independently by two blinded investigators who scored maximal observable co-localization among all cells at an arbitrary value of 5. Each cell was then scored relative to that. The mean \pm standard deviation is shown.

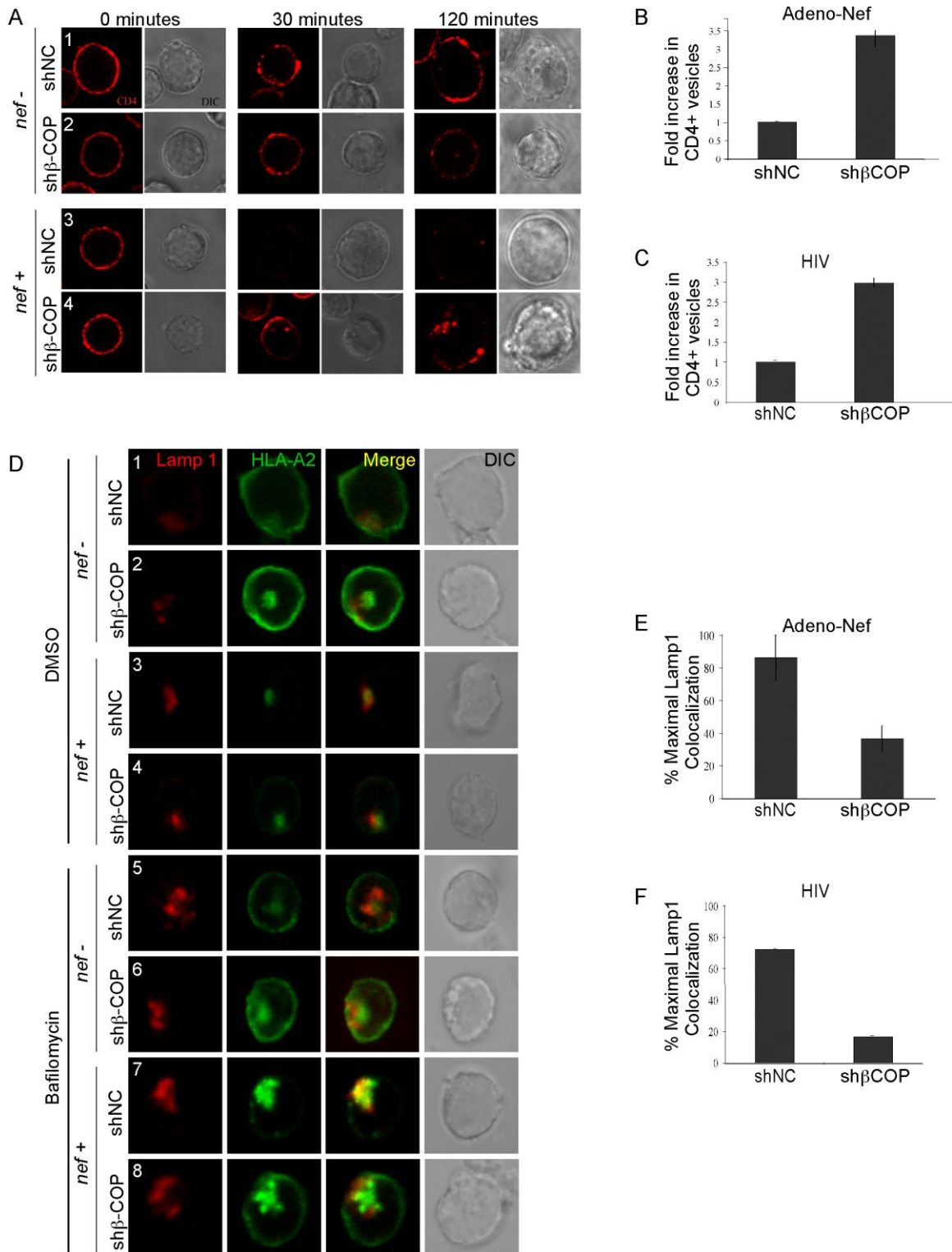


Figure 3.8. Nef requires β -COP to target HLA-A2 and CD4 for degradation.

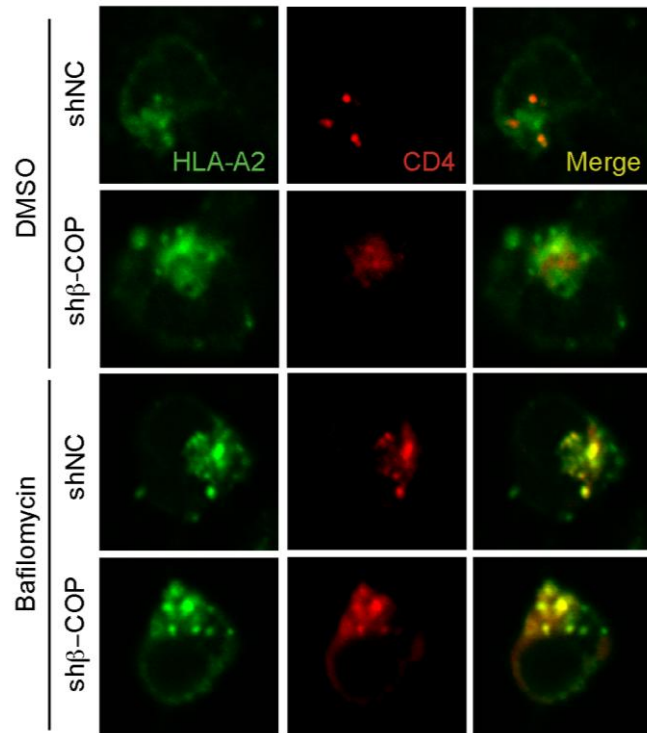


Figure 3.9. Sh β -COP does not disrupt co-localization of CD4 and HLA-A2, but does increase the amount of stainable protein within the cell.

HLA-A2 CEM cells were transduced with a lentivirus expressing control (shNC) or β -COP (sh β -COP) shRNA. After 3 days, the cells were transduced with adeno-Nef. After three additional days, the cells were stained with antibodies directed against HLA-A2 and CD4 as in Figure 3.3. Images were taken with an Olympus FV-500 confocal microscope and processed with Adobe Photoshop software. Single Z-sections are shown.

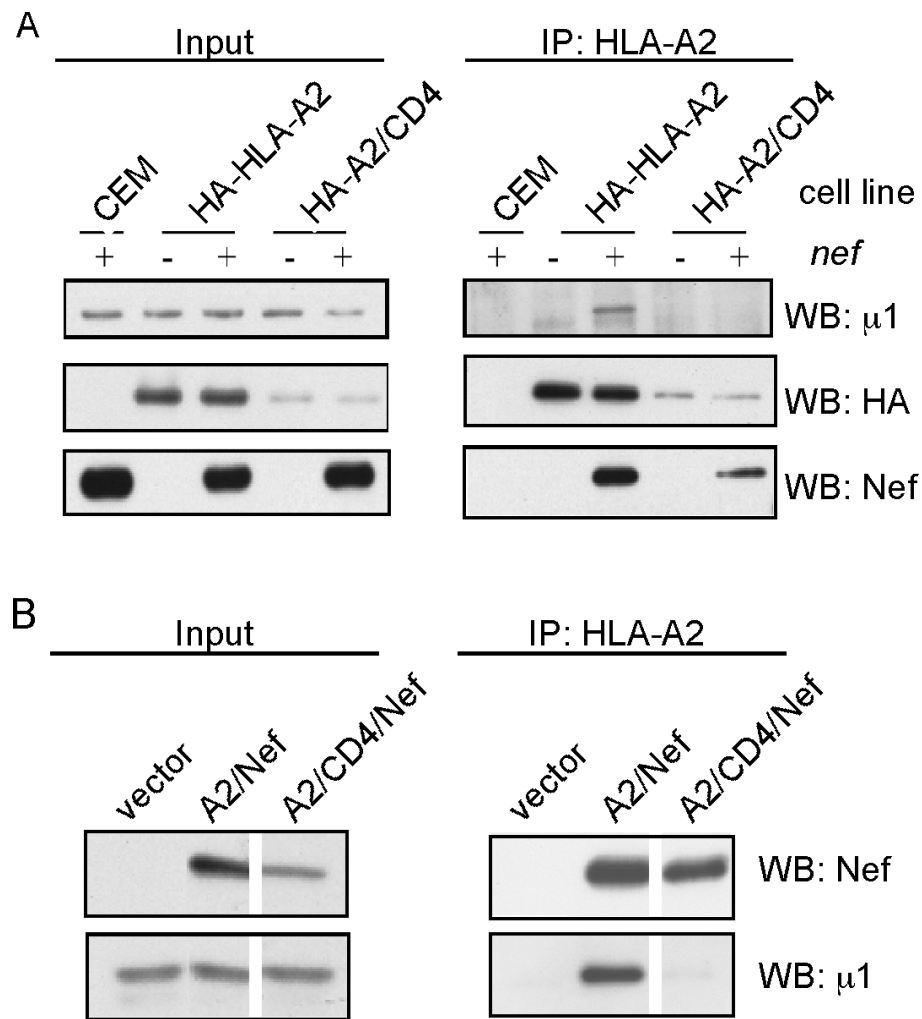


Figure 3.10. Selective binding of AP-1 is dependent on the cytoplasmic tail.

(A) The HLA-A2 cytoplasmic tail is necessary for co-precipitation of AP-1. Parental HLA-A2-negative CEM T cells (CEM) or CEM T cell lines expressing HA-HLA-A2 or HA-A2/CD4 were transduced with adeno-Nef or a control adenovirus. Lysates were immunoprecipitated with an antibody directed against HLA-A2 (BB7.2) and the presence of Nef or AP-1 was detected by western blot analysis. Results are representative of three independent experiments. (B) The cytoplasmic tail is necessary for the HLA-A2/Nef fusion protein to co-precipitate AP-1 in Nef expressing T cells. CEM T cells were transduced with a murine retroviral vector expressing no protein (vector), A2/Nef or A2/CD4/Nef fusion proteins. These cells were immunoprecipitated with an anti-HLA-A2 antibody (BB7.2) and western blot analysis was performed to detect co-precipitation of AP-1. Spaces between lanes indicate where intervening lanes were cropped out to remove irrelevant data. Results are representative of two independent experiments.

Figure 3.11. Co-precipitation of Nef and β -COP depends on domains of Nef that are also needed for MHC-I downmodulation. (A) Flow cytometric analysis of Nef mutants defective at MHC-I downmodulation. CEM T cells treated with control adenovirus (nef^-), adeno-Nef (nef^+) or the indicated mutant were stained either with an anti-HLA-A2 antibody (BB7.2) or an antibody directed at CD4. Cells were analyzed by flow cytometry as described in Materials and Methods. (B) Quantitation of MHC-I and CD4 downmodulation by Nef and Nef mutants. Fold downmodulation was determined by dividing the mean fluorescence intensity (MFI) of control virus treated cells by the MFI of Nef-expressing cells. The average value from three (wild-type) or two (mutant Nef) experiments was plotted \pm the standard deviation. (C) Nef D_{123}G and $\text{V}_{10}\text{E}\Delta 17-26$ mutants are defective at β -COP binding. CEM T cells were treated with control adenovirus (nef^-), adeno-Nef (nef^+), or the indicated mutant and immunoprecipitated with a control antibody (BB7.2) or an antibody directed against β -COP (M3A5). The presence of Nef was detected by western blot analysis. Arrows indicate the positions of wild type Nef and Nef $\text{V}_{10}\text{E}\Delta 17-26$. Results are representative of at least two independent experiments. (D) $\text{V}_{10}\text{E}\Delta 17-26$ Nef is defective at MHC-I, but not CD4, degradation. CEM cells expressing HA-HLA-A2 and HA-A2/CD4 were transduced with adeno-viral vectors encoding wild-type Nef (Nef^+), $\text{V}_{10}\text{E}\Delta 17-26$ Nef, or a control adenoviral vector (Nef^-). Two days later, the media on half of the cells was replaced with media containing 20 mM ammonium chloride to inhibit lysosomal degradation. The next day, the cells were harvested, lysed, and normalized. Each sample was split equally and one set was treated with endo H. Protein levels of HA-HLA-A2 and HA-A2/CD4 were assessed by western blot analysis using an anti-HA antibody. Endo H-resistant bands are marked with an R and endo H-sensitive bands are marked with an S.

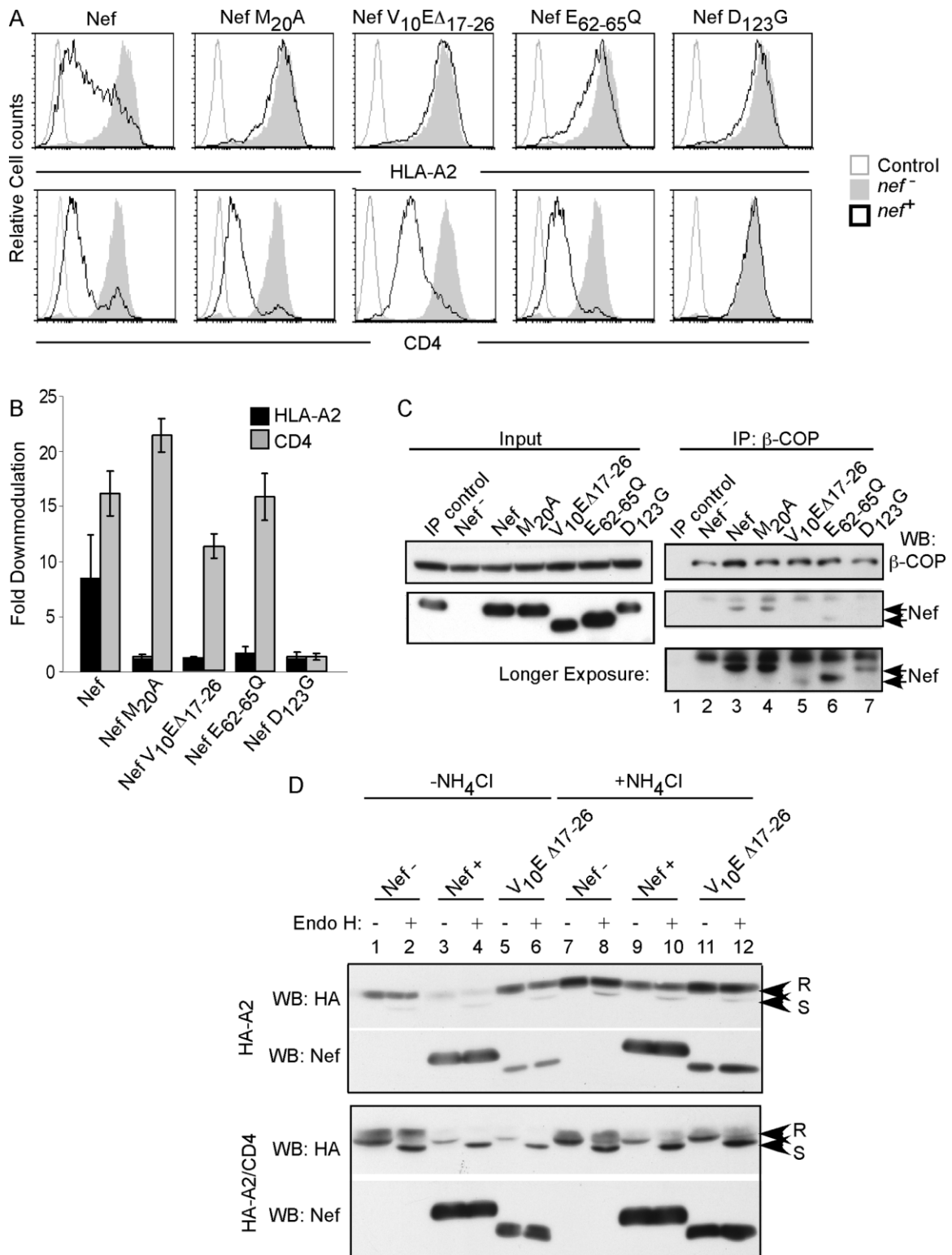


Figure 3.11. Co-precipitation of Nef and β-COP depends on domains of Nef that are also needed for MHC-I downmodulation.

Figure 3.12. Two Nef domains recruit β -COP, but only one is used for the degradation of HLA-A2. (A) Flow cytometric analysis of HLA-A2 and CD4 expression in cells expressing Nef mutants. CEM T cells stably expressing HLA-A2 were spin-transduced with murine retroviral supernatants that express the indicated Nef construct and a GFP cassette from an internal ribosomal entry site. The cells were gated for GFP expression and results of HLA-A2 (top panel) or endogenous CD4 (bottom panel) staining are shown. R/E stands for R_{17,19}A/E_{154,155}A double mutant. Open light gray curve, parental cell line; shaded dark gray curve, empty vector; black shaded curve, wild type Nef; and open dark gray curve, Nef mutant. (B) Quantification of down-modulation. The mean \pm SD for greater than or equal to six experiments (actual number varies depending on the mutant) is shown. (C,D) R_{17/19} is needed for optimal HLA-A2 degradation, whereas the E_{154/155} is dispensable. CEM T cells expressing HLA-A2 and Nef were generated as described in part A. The cells were pulse labeled with ³⁵S-labeled amino acids, chased for 0 or 12 hours in complete medium and lysed. HLA-A2 was immunoprecipitated with the anti-HLA-A2 antibody BB7.2, separated by SDS-PAGE and quantified using a phosphorimager. “Ig Control” indicates results from HLA-A2-negative parental CEM cells immunoprecipitated with BB7.2 antibody. (D) Quantification of degradation. Nef activity was calculated as follows: (the fraction of HLA-A2 remaining in control cells /the fraction of HLA-A2 remaining in Nef expressing cells). The value obtained for each mutant was divided by that for wild type Nef and multiplied by 100 to calculate % wild type activity. The mean \pm SD for two experiments is shown. (E) R_{17/19} and E_{154/155} are required for the β -COP/Nef interaction. CEM T cells expressing HA-A2 were transduced with a retroviral vector expressing either wild-type Nef or the indicated Nef mutant. The cells were immunoprecipitated with an anti- β -COP antibody and the presence of Nef was assessed by western blot as described in Materials and Methods. The Ig control is HLA-A2-negative parental CEM cells expressing wild-type Nef immunoprecipitated with a control antibody (BB7.2) and the vector only control is CEM cells expressing HLA-A2 transduced with the empty retroviral vector.

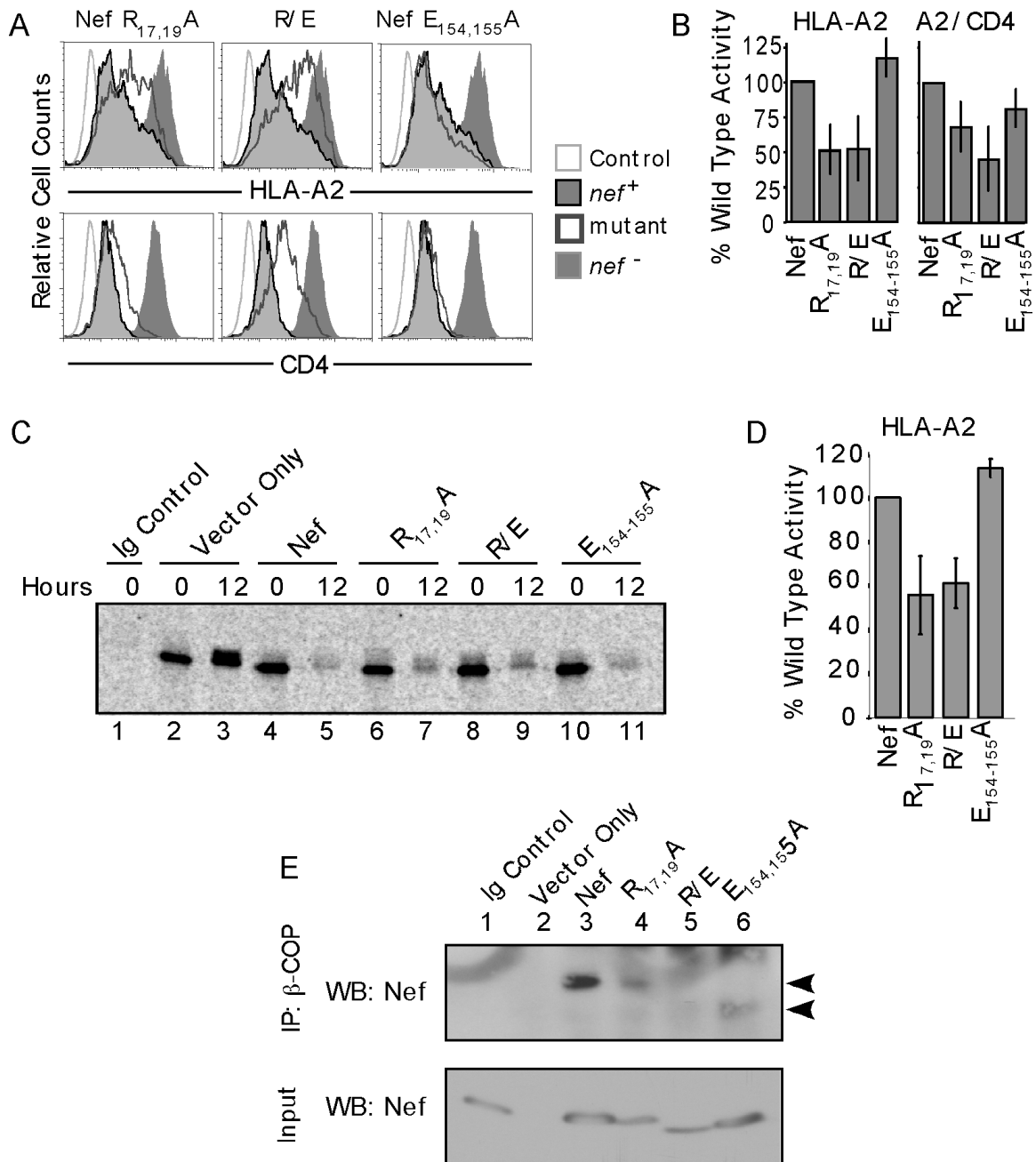


Figure 3.12. Two Nef domains recruit β -COP, but only one is used for the degradation of HLA-A2.

Figure 3.13. Nef uses the E_{154/155} to promote maximal CD4 degradation. (A) Cells expressing HA-HLA-A2/CD4 were treated as in Figure 3.12A, lysed and treated with endo H as indicated. The samples were separated by SDS-PAGE and western blotted for the HA tag on HA-A2/CD4. (B) Quantification of degradation. Western blots were quantified using Adobe Photoshop software. Nef activity was calculated as follows (fraction of total protein that was endo H-resistant for wild type Nef/fraction endo H-resistant for each mutant)×100. The mean±SD for four experiments is shown. (C) Model for the mechanism by which Nef affects CD4 and MHC-I trafficking. HIV Nef binds the CD4 cytoplasmic tail at the cell surface, and recruits AP-2 and/or the vacuolar-ATPase to facilitate internalization. CD4 is internalized and is transported to an endosomal compartment associated with Rab7 and β-COP. In contrast, Nef binds the MHC-I cytoplasmic tail early in the secretory pathway, AP-1 is recruited and facilitates transport to an intermediate endosomal compartment marked with Rab7. If AP-1 falls off the Nef-MHC-I complex after arrival in the endosome, Nef binds β-COP and targets MHC-I (and CD4) to lysosomes for degradation. If AP-1 remains bound, it promotes recycling of the Nef-MHC-I complex to the TGN. LY = lysosome, LE/MVB = late endosome/multi-vesicular body.

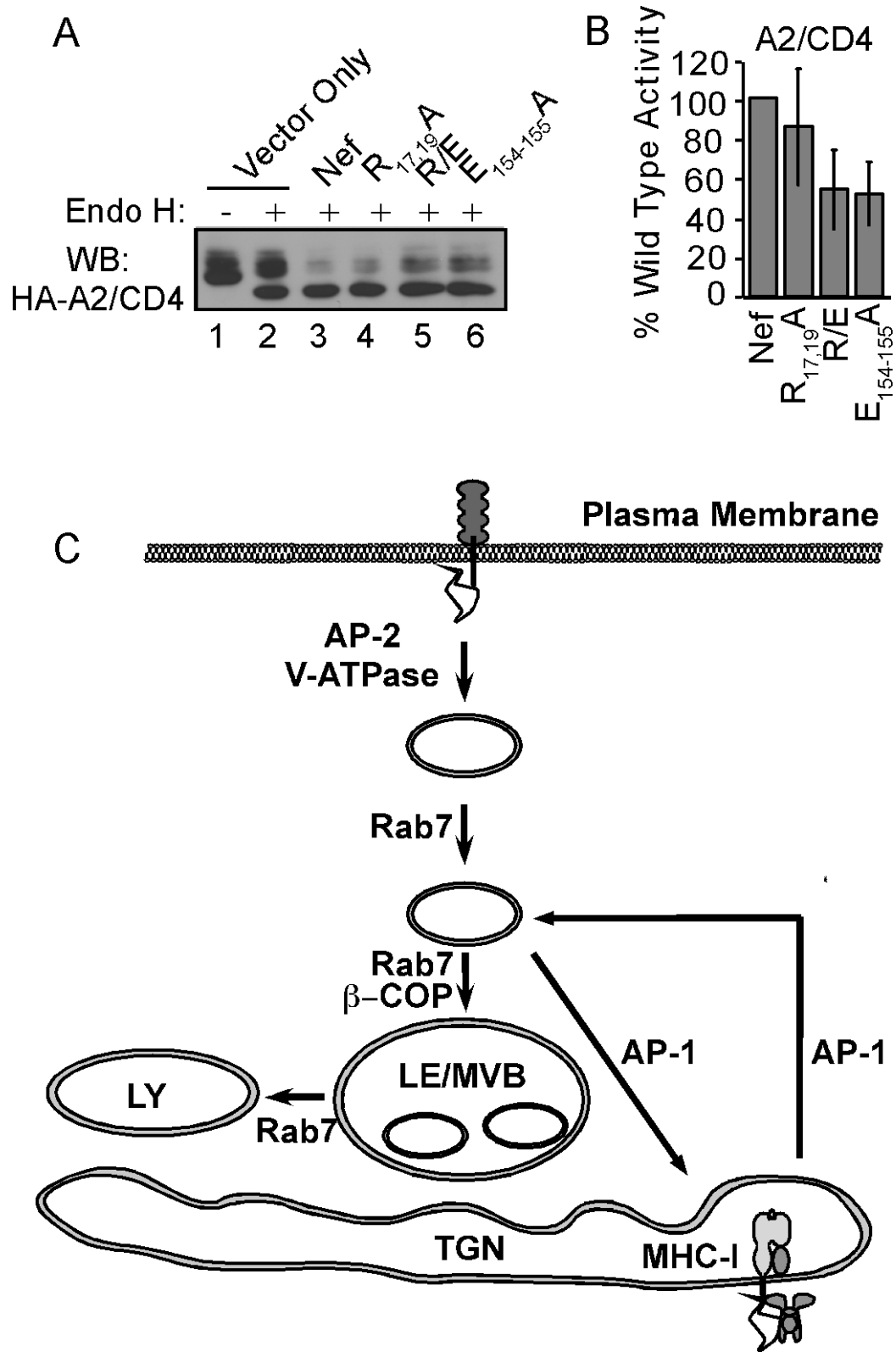


Figure 3.13. Nef uses the E_{154/155} to promote maximal CD4 degradation.

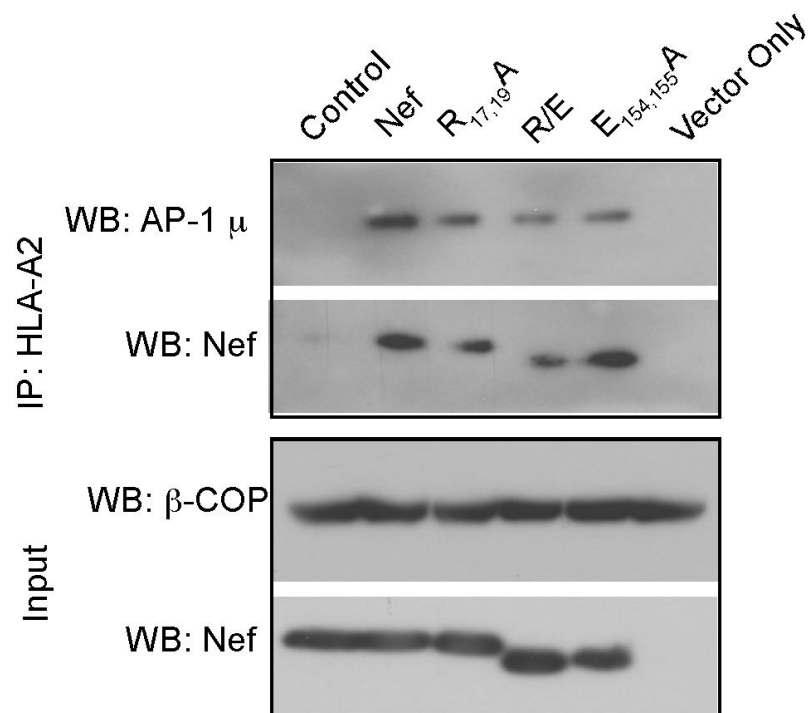


Figure 3.14. Mutation of R_{17/19} and E_{154/155} only slightly diminishes the amount of Nef and AP-1 coprecipitating with HLA-A2.

CEM cells expressing HA-HLA-A2 were transduced with a retroviral vector expressing either wild type Nef or the indicated Nef mutant. The cells were immunoprecipitated with an anti-HLA-A2 antibody (BB7.2), and the presence of Nef was assessed by Western blot as described in Materials and Methods. “Control” indicates lysates from parental CEM T cells that lack HLA-A2, but that express wild-type Nef. “Vector only” indicates CEM T cells expressing HA-A2 transduced with empty retroviral vector.

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CHAPTER IV

HIV-1 Nef requires functional ARF-1 to downmodulate MHC-I

Abstract

HIV-1 Nef downmodulates major histocompatibility complex (MHC) class I molecules in order to evade recognition by the host's immune response. Nef has been shown to bind the cytoplasmic tail of the MHC-I allotype HLA-A2 and to recruit the clathrin adaptor protein AP-1, which re-directs MHC-I to endolysosomal compartments, and promote MHC-I degradation. We explored whether this process bypassed normal cellular mechanisms that govern AP-1 activity. We found that MHC-I downmodulation by Nef was unaffected by mutation of a putative phosphorylation site (T₁₅₄) in AP-1 that we confirmed was needed in other contexts. In contrast, Nef-dependent MHC-I trafficking did require active ARF-1, a GTPase normally required for AP-1 activity. The dominant active ARF-1 mutant (ARF-1 Q₇₁L) co-immunoprecipitated with the AP-1-MHC-I-Nef complex, and appeared to stabilize it by disallowing coatomer release. Correspondingly, brefeldin A, which inhibits ARF-1, disrupted formation of Nef-MHC-I-AP-1 complex and inhibited MHC-I downmodulation. In contrast, mutation of ARF-6, which has been implicated in MHC-I internalization by Nef had no effect on MHC-I surface expression levels. These data support a model in which Nef and ARF-1 stabilize an interaction between MHC-I and AP-1 to disrupt MHC-I trafficking to the cell surface.

Introduction

HIV-1 evades immune system recognition by inhibiting viral antigen presentation through major histocompatibility complex (MHC) class I downmodulation (1,2). MHC-I downmodulation and protection from CTL recognition depends on expression of the HIV-1 Nef accessory protein. Nef is a multifunctional adaptor protein that binds directly to the cytoplasmic tail of HLA-A and HLA-B MHC-I allotypes (3) and recruits the clathrin adaptor protein, AP-1, to redirect MHC-I into an endolysosomal pathway from the TGN and to prevent its expression at the cell surface (4). There is evidence that MHC-I delivered to endosomes via the activity of AP-1, is subsequently targeted for degradation via Nef binding to the COP-I coatomer, β -COP (5). Nef has also been implicated in promoting internalization of MHC-I from the cell surface via an ARF-6- (6) or an AP-1 (7,8) dependent pathway at least in some cell systems (9). However, the relative contribution of these pathways on cell surface MHC-I expression has not been directly examined in the same cell system.

The adaptor protein, AP-1, is a heterotetrameric complex that recognizes trafficking signals in cargo and recruits clathrin sorting machinery to the *trans*-Golgi network. AP-1 is made up of a μ 1, β 1, γ , and σ 1 subunit which collectively sort cargo containing Yxx ϕ or [D/E]xxxLL trafficking signals into the endo-lysosomal network (for review see (10)). MHC-I downmodulation can be inhibited by knocking down expression of the AP-1 μ 1 subunit (4,5) or by overexpressing a dominant negative mutant of AP-1 μ 1 which lacks a functional tyrosine binding pocket (11,12).

General phosphorylation of the α , β 2, and μ 2 subunits in AP-2 increases its affinity for Yxx ϕ , dileucine-based, and non-canonical sorting signals (13). In particular, phosphorylation of AP-2 μ 2 subunit increases AP-2 binding to a Yxx ϕ sorting signal in the cytoplasmic tail of lysosomal acid phosphatase (LAP) (14). Additionally, a phosphorylation site on the μ 2 subunit (T₁₅₆) is required for endocytosis of transferrin (15-17). Similarly, AP-1 μ 1 isolated from membrane preparations in [³²P]orthophosphate labeled cells was phosphorylated while cytosolic AP-1 μ 1 was unlabeled (18). Clathrin coated vesicle preparations of AP-1 were able to bind to a GST-fused mannose-6-

phosphate receptor (MPR) cytoplasmic tail, while cytosolic AP-1 was unable to interact with the same sorting signal (18). Finally, AP-1 complexes isolated from clathrin coated vesicles treated with the phosphatase PP2A were unable to interact with the MPR sorting signal (18). The combination of these pieces of data and the correlations between the crystal structure of AP-1 and AP-2 (19,20) suggests that phosphorylation of AP-1 increases AP-1's affinity for Yxx ϕ sorting signals *in vitro* (18,21). The effect of AP-1 μ 1 phosphorylation has yet to be examined functionally with regard to intracellular trafficking and Nef-induced AP-1-dependent trafficking.

ADP-ribosylation factors (ARFs) are small GTPases that control assembly and disassembly of various intracellular trafficking complexes. ARF activation and recruitment to cellular membranes is cyclical and regulated by its GTP binding state. Guanine nucleotide exchange factors (GEFs) promote the exchange of GTP for GDP. GTPase-activating proteins (GAPs) support ARF catalysis of GTP, and thus are important to inactivate ARF ((22) and reviewed in (23)). Specifically, ARF-1 is a clathrin regulatory protein that upon binding GTP undergoes a conformational change exposing a myristoyl group that inserts into membranes and subsequently stabilizes AP-1 (24-26) or COP-I coatomer (26-28) recruitment to appropriate signals. The ARF-1 GEF inhibitor, brefeldin A, stabilizes an abortive ARF-GDP-bound complex (29) thus preventing ARF-1 cycling. Pan ARF-1 GEF inactivation causes Golgi dissociation, thus suggesting functional ARF-1 is required for maintenance of Golgi structure (30). ARF-6 localizes to the plasma membrane and is involved in clathrin-independent endocytosis (31,32). Dominant inhibitory mutants of ARFs have been shown to be locked in either a GDP-bound, inactive state (ARF-1-T₃₁N or ARF-6-T₂₇N) or a GTP-bound, active state (ARF-1-Q₇₁L or ARF-6-Q₆₇L) (31-34).

Nef downmodulates the viral co-receptor CD4 to inhibit superinfection (35) and promote viral assembly and release (36,37). In Nef expressing cells, CD4 transports through the secretory pathway normally (38,39) and is rapidly internalized into endosomal compartments (40). Previous research has shown that ARF-1 is involved in COP-I coatomer recruitment to the endocytosed CD4-Nef complex (41) resulting in CD4 localization to acidic compartments (42). However, it is not known whether ARF-1 is needed for Nef to recruit AP-1 to the cytoplasmic tail of MHC-I.

In this manuscript, we demonstrate that Nef appears to bypass the need for AP-1 μ 1 phosphorylation to downmodulate the MHC-I allotype, HLA-A2. However, we found that inhibiting ARF-1 with brefeldin A (BFA) or using the dominant active Q₇₁L molecule inhibited Nef-dependent MHC-I trafficking. Moreover, AP-1 recruitment to the MHC-I-Nef complex was specifically inhibited by brefeldin A (BFA) and stabilized by ARF-1 Q₇₁L. In contrast, we were unable to detect any requirement for ARF-6.

Materials and Methods

Cell Lines. CEM-SS T4 lymphoblastoid, Bosc (43), and 293T cell lines were cultured as described previously. CEM cells expressing HA-Tagged HLA-A2 and HA-HLA-A2-A₃₂₃V (HLA-A2 A₃₂₃V) were created and maintained as previously published (11).

DNA Constructs. FG12 shRNA Lentiviral vectors against either a negative control or AP-1 μ 1A were previously published (5).

Construction of a vector that expresses placental alkaline phosphatase (PLAP) from an internal ribosomal entry site (IRES). A PLAP open reading frame (ORF). was amplified through PCR to contain XcaI and SalI restriction sites. The digested PLAP cassette was cloned into a shuttle vector pCITE that had been cut with MscI and SalI. To maintain novel restriction sites, PLAP was then excised from pCITE with EcoRI digestion. Excised PLAP was ligated into an EcoRI digested murine stem cell virus (MSCV) that already contained an IRES and a puromycin selection cassette (MSCV-IRES-Puro) to generate MSCV IRES PLAP. Utilized below, an IRES-PLAP cassette was prepared by digesting MSCV IRES PLAP with EcoRI and XhoI restriction enzymes.

Construction of AP-1 μ R and AP-1 μ R mutants. The human AP-1 μ 1A ORF was amplified from the MegaMan human transcriptome library (Stratagene) with 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 (11). Silent mutations were introduced in two regions of AP-1 μ 1A to create AP-1 μ Rescue (μ R), which cannot be recognized by AP-1 μ 1A shRNAs we designed previously (5). Standard two-round, PCR mutagenesis was performed on the AP-1 μ 1A insert to mutate the first shRNA recognition site using 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 and the following sense and anti-sense oligos to generate an AP-1 μ 1A-Site 1 Rescue insert: site 1 sense primer 5'-AAGAATGAGGTCTTTCTCGATGTGATCGAG-3'. A second round of two-step mutational PCR was performed on the AP-1 μ 1A-Site 1 Rescue insert to mutate the second shRNA recognition site using 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 and the following sense and anti-sense oligos to generate a wild type AP-1 μ R insert: site 2 sense primer 5'-CTCAACGATAAAGTGTTGTTTCG-ATAACACG-3'.

Silent mutations were introduced into AP-1 μ IR to create a tyrosine binding pocket mutant (AP-1 μ R TBPM) through two step PCR mutagenesis using 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 and a different site 1 primer as F₁₇₂ and D₁₇₄ were contained in the shRNA target sequence. The TBPM site 1 sense primer has the following sequence: 5'-AAGAATGAGGTCGCCCTCTCCGTG-ATCGAG-3'.

AP-1 μ R T₁₅₄A was created through two step PCR mutagenesis by amplifying wild type AP-1 μ R with 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 and the following sense and anti-sense oligos: 5'-CCACCAGCCACCGTCGC~~CA~~AACGCGGTGTCCTGG-3'. T_{152/154}A was created through two step PCR mutagenesis by amplifying wild type AP-1 μ R with 5'BamHIKozakAP-1 and 3'EcoRIstopAP-1 and the following sense and anti-sense oligos: 5'-CCGCGGCCACCAGCCG~~CC~~CGTCGC~~CA~~AACGCGGTGTCC-TGG-3'.

Each AP-1 μ R insert, digested with BamHI and EcoRI restriction enzymes, and an IRES PLAP cassette, digested with EcoRI and XhoI, were ligated into BglII and XhoI digested MSCV Puro (44), thus creating MSCV AP-1 μ R IRES PLAP, MSCV AP-1 μ R TBPM IRES PLAP, MSCV AP-1 μ R T₁₅₄A IRES PLAP, MSCV AP-1 μ R T_{152/154}A IRES PLAP. This allowed for puromycin selection of cell lines stably expressing each AP-1 μ R cassette.

Construction of MSCV IRES GFP vectors expressing ARF-1 and ARF-6. pCB6 expressing Myc-tagged wild type or T₃₁N ARF-1 were obtained from Didier Trono (Ecole Polytechnique Fédérale de Lausanne) and pXS expressing HA-tagged wild type, T₂₇N, or Q₆₇L ARF-6 was obtained from Julie Donaldson (National Institutes of Health). The ARF-1 open reading frame was amplified using either wild type or T₃₁N pCB6 Myc-ARF-1 as a template and using the following primers: open reading frame (ORF) start sense primer 5'-CGCGGATCCGCCACCATGGGGAACAT-CTTCGCC-3' and Myc-tag antisense primer 5'-CGCGGATCCTCATAGATCTTCTTC-TGAGAT-3'. Standard techniques were used to clone the PCR product into the BamHI site of MSCV IRES GFP (pMIG) (45) creating MSCV Myc-ARF-1 IRES GFP and MSCV Myc-ARF-1 T₃₁N IRES GFP. MSCV Myc-ARF-1 Q₇₁L IRES GFP was created through standard two-step PCR mutagenesis using wild type MSCV ARF-1 IRES GFP as a template using the following sense primer: 5'-TGGGACGTGGGTGGCCTGGACAAGATCCGGCCC-3'. ARF-6 was amplified using (pXS HA-ARF-6) as a template and using the following primers:

ORF start sense primer 5'-GAAGATCTGCCACCATGGGGAAGGTGCTATCC-3' and ORF stop and HA-tag antisense primer 5'-GCGAATTCTTAAGCGTAATCTGGAACA-TCGTA-3'. Standard techniques were used to clone the PCR product into Bgl II and EcoRI sites of pMIG creating MSCV ARF-6 IRES GFP, MSCV ARF-6 T₂₇N IRES GFP, and MSCV ARF-6 Q₆₇L IRES GFP. DNA constructs were transfected into Bosc or 293T cells with linear polyethylenimine, MW 25,000 Da (Polysciences Incorporated). Supernatants from transfected cells were harvested and used to transduce CEM T cells.

Virus Preparation and Transductions. High-titer Lentivirus containing shRNA was produced as previously described (46). Briefly, 293 cells were transfected with the FG12 constructs previously described (5), pRRE (47), pRSV-Rev (47), and pHCMV-G (48). 1×10^5 CEM T cells were transduced with 1 ml of viral supernatant with 8 μ g/ml Polybrene at 2500rpm for two hours in a tabletop centrifuge.

Retroviral supernatants using a bi-cistronic retroviral vector expressing an IRES GFP cassette (pMIG) were prepared as described previously (43,45). Bosc cells (43) were transfected with MSCV constructs described above, the retrovirus packaging vector pCL-Eco (49), and pHCMV-G (48). Briefly, 5×10^5 CEM cells were spin-transduced in 1 ml of retroviral supernatants with 8 μ g/ml Polybrene at 2500rpm for two hours in a tabletop centrifuge.

Replication defective adenovirus was produced by the University of Michigan Gene Vector Core facility. Adenoviral transductions were performed as previously described (50). Briefly, transductions were performed as follows: 1×10^6 CEM-SS cells in 1 mL of RPMI 1640 containing 2% fetal bovine serum, 10mM HEPES, and 2mM penicillin, streptomycin, and glutamine. Multiplicity of infection was 200 (based on 293 cell infectivity, which is greater than CEM infectivity). Low serum transductions ranged from 4-6 hours.

Flow cytometry and Antibodies. Cells were stained for 20 minutes on ice in FACS buffer (PBS, 1% Human Serum, 1%FBS, 1% HEPES, and 1% NaN₃, washed and then stained for 20 additional minutes in secondary antibody. Mouse antibody to HLA-A2 antibody, BB7.2 (51) and mouse antibody to CD4, OKT4 (52) were purified from ascites (9) provided by the University of Michigan Hybridoma core facility. Mouse antibody to PLAP antibody was obtained from Serotec. Secondary antibodies used were:

goat anti-mouse IgG_{2B}-phycoerythrin (Invitrogen, 1:250) and goat anti-mouse IgG₁-Alexa Fluor 647 (Invitrogen, 1:250). Isotype controls were obtained from BD Biosciences. Stained cells were analyzed on a Becton Dickinson FACSCanto cytometer. Analysis was performed using FlowJo software (Tree Star Inc.).

Immunoprecipitations and Western Blotting. Immunoprecipitations were performed as previously described (11). 15×10^6 CEM cells were transduced with either control or Nef-expressing Adenovirus. At 48 hours post-Adenoviral transduction, the cells were spin-transduced with either DMEM (mock) or MSCV ARF-1 IRES GFP viral supernatants. At 72 hours post-Adenoviral transduction, all cells were incubated in 20mM NH₄Cl for 16 hours. Where indicated, samples were also incubated in 50 μ M BFA for 16 hours. Cells were then harvested and lysed in 1% digitonin (Wako) lysis buffer described previously (5,11). Lysates were normalized for total protein and GFP transduction rates, when appropriate, prior to immunoprecipitation. Input controls were 1% of the immunoprecipitated protein. After an overnight pre-clear at 4°C, lysates were immunoprecipitated for either HLA-A2 (BB7.2 crosslinked beads) (11) or Myc-tagged ARF-1 (9e10 (53) crosslinked beads). Immunoprecipitates were eluted and analyzed by western blot as previously described (50). Western blot antibodies used were anti-Nef (AG11, (54)) and anti-Myc (9e10, (53)), which were produced by the University of Michigan Hybridoma Core facility and purified as previously described (9). Antibodies also used for western blotting were anti-AP-1 γ (BD Biosciences), HA (HA.11, Covance) anti-AP-1 μ 1 (RY/1, (55)). The secondary antibody for anti-Nef, anti-HA, and anti-Myc was goat anti-mouse IgG-HRP (Zymed Laboratories Inc). The secondary antibody for anti-AP-1 μ 1 was goat anti-rabbit-HRP (Zymed Laboratories Inc). The secondary antibody for AP-1 γ was rabbit anti-mouse-HRP (Zymed Laboratories Inc).

Results

Putative phosphorylation sites in AP-1 μ 1 are not required for MHC-I downmodulation by Nef. Functional studies of AP-2 have revealed that phosphorylated AP-2 μ 2 is required for AP-2 localization to clathrin coated pits and endocytosis of cargo proteins (15-17). Specifically, a threonine in AP-2 μ 2 (T₁₅₆) has been identified as a critical phosphorylation site that once mutated inhibits endocytosis of transferrin (15,17). Similarly, studies have revealed that phosphorylated AP-1 μ 1 associates with membranes and cargo signals (18). The crystal structure of AP-1 (20) and AP-2 (19) have been solved and distinct similarities between the two structures reveal a putative phosphorylation site on AP-1 μ 1 (T₁₅₄) and a second threonine in close proximity (T₁₅₂).

To determine if phosphorylation of AP-1 μ 1 is required for Nef to downmodulate HLA-A2 we examined whether the putative phosphorylation site (T₁₅₄A) and in addition the neighboring threonine (T_{152/154}A) in AP-1 μ 1 were required for this process.

We first examined this question using an MHC-I HLA-A2 in which the YSQA sequence in the HLA-A2 cytoplasmic tail was changed to a Yxx ϕ sorting signal, Y₃₂₀SQV₃₂₃ (HLA-A2 A₃₂₃V) (11). This molecule was previously shown to have low expression at the cell surface due to its interaction with AP-1. Consistent with this, we observed an increase in surface expression of HLA-A2 A₃₂₃V in response to AP-1 knockdown. This effect was not due to off-target effects of the AP-1 siRNA because the cell surface phenotype was reversed with expression of an AP-1 with silent nucleotide substitutions in the siRNA binding region (AP-1 μ R), (Figure 4.1A, quantified in 1C). A representative western blot revealing AP-1 μ 1 knock down and recovery of expression with AP-1 μ 1R is shown in Figure 4.1E. In contrast, both AP-1 μ R with a mutation in the putative phosphorylation site T₁₅₄ and AP-1 μ R with a mutation in the tyrosine binding pocket ((TBPM) (11)) failed to rescue knock down. Surprisingly, a combination mutant that disrupted two threonine residues in close proximity (T_{152/154}) rescued knock down, (Figure 4.1A, quantified in 1C) suggesting a complex interplay between these amino acids. As expected, knockdown of AP-1 had no effect on HLA-A2 molecules that lacked an AP-1 signal (quantified in Figure 4.1D, left chart).

We then asked about the requirement for T₁₅₄ in Nef-mediated MHC-I downmodulation. As previously shown, knockdown of endogenous AP-1 μ 1 reversed downmodulation of MHC-I Nef (4,7) and overexpression of AP-1 μ R rescued this phenotype. In contrast, AP-1 μ R with a mutation in the tyrosine binding pocket (TBPM) failed to rescue the Nef phenotype and indeed increased surface expression even more than shAP1 alone. Finally, AP-1 μ R with mutation in the putative phosphorylation sites (T₁₅₄A or T_{152/154}A) rescued the Nef phenotype to the same extent as AP-1 μ R indicating that there was no role for these amino acids in Nef-dependent MHC-I downmodulation.

Functional ARF-1 is required for Nef to disrupt the trafficking of MHC-I.

We next examined whether the GTPase, ARF-1, which is normally required for AP-1 function is necessary for Nef to disrupt the transport of MHC-I to the cells surface. To do this, we utilized wild type, dominant negative (T₃₁N), and dominant active (Q₇₁L) ARF-1 molecules. We also tested brefeldin A (BFA), which inhibits the activity of the ARF-1 GTP exchange factor (GEF).

We found that expression of wild type and T₃₁N ARF-1 did not significantly affect Nef-dependent downmodulation of HLA-A2, HLA-A2 A₃₂₃V or CD4 (Figure 4.2A, quantified in Figure 4.2B). However, inhibition of ARF-1 with Q₇₁L ARF-1 expression or BFA treatment significantly reduced Nef activity on MHC-I HLA-A2 (Figure 4.2A, quantified in Figure 4.2B and C) and on HLA-A2 A₃₂₃V (Figure 4.2A, quantified in Figure 4.2B and C). BFA treatment in the absence of Nef reduced the surface expression of HLA-A2, HLA-A2 A₃₂₃V and CD4, (quantified in figure 4.2C) presumably because of the effects of BFA on export of proteins from the endoplasmic reticulum (56). However, CD4 downmodulation by Nef was unaffected by BFA treatment consistent with that fact that this process is independent of AP-1 (Figure 4.2A, quantified in Figure 4.2C).

Because both BFA and ARF-1 T₃₁N should act as negative inhibitors of ARF-1, it is unclear why, BFA blocked Nef activity, but ARF-1 T₃₁N did not. The most likely explanation is that we were unable to achieve high enough levels of ARF-1 T₃₁N for it to behave as a dominant negative inhibitor of endogenous wild type ARF-1.

Similar to the results we obtained with BFA, we noted a striking inhibitory effect of dominant active ARF-1 (Q₇₁L) on Nef activity (Figure 4.2A, quantified in Figure

4.2B). In a previous report, quantitation of electron microscopy revealed that vesicles were unable to uncoat in cells overexpressing GTP-bound Q₇₁L ARF-1 or cells incubated in the presence of nonhydrolyzable GTP [GTP γ S] (57). Based on these results, we speculated that the inhibitory effect of ARF-1 Q₇₁L was due to an inability of the Nef-MHC-I-AP-1 complex to efficiently disassemble.

We also examined the potential role of another GTPase, ARF-6, which has been implicated in the Nef-dependent internalization of MHC-I (6). For these experiments, we utilized wild type, dominant negative (T₂₇N), and dominant active (Q₆₇L) molecules. As seen by surface expression analysis in Figure 4.3A and B (quantified in Figure 4.3C and D), we observed no effect of these mutants on Nef-mediated MHC-I or CD4 downmodulation. Western blot confirmed that these proteins were appropriately expressed (Figure 4.3, panel C). Additionally, expression of the ARF-6 IRES GFP construct was monitored by GFP expression and was similar to that obtained by the ARF-1 IRES GFP constructs used in Figure 4.2 (data not shown). These data indicate that ARF-6-dependent MHC-I internalization does not play a significant role in MHC-I downmodulation by Nef in our system.

Dominant active ARF-1 increases AP-1 recruitment to HLA-A2 A₃₂₃V and the HLA-A2-Nef complex. To determine the mechanism by which ARF-1 affects MHC-I surface expression, we used co-immunoprecipitation assays to measure AP-1 binding to cargo plus or minus expression of ARF-1 mutants. First we examined AP-1 binding to a Yxx ϕ sorting signal using HLA-A2 Y₃₂₀SQV (HLA-A2 A₃₂₃V) in the absence of Nef. As seen in Figure 4.4 lanes 2, 3, 4, and 5, and as previously reported (11), AP-1 co-precipitated with this molecule in the absence of Nef. Expression of wild type (lane 3) or T₃₁N ARF-1 (lane 5) did not affect AP-1 recruitment by this assay. However expression of dominant active ARF-1 Q₇₁L (lane 4) caused a dramatic enhancement of AP-1 recruitment to HLA-A2 A₃₂₃V.

Next, we used the same assay system to determine whether ARF-1 activity affected Nef-dependent recruitment of AP-1 to wild type HLA-A2. Similar to what was observed with HLA-A2 A₃₂₃V, we found that dominant active ARF-1 Q₇₁L increased co-precipitation of AP-1 with HLA-A2 in Nef expressing cells, whereas treatment with BFA disrupted it (Figure 4.4B). The effect of BFA was specific, because it could be rescued

by overexpression of ARF-1 Q₇₁L as previously reported (58). Similar to what we observed with HLA-A2 A₃₂₃V, expression of ARF-1 T₃₁N had little effect on AP-1 co-precipitation (Figure 4.4, lane 9).

We were also able to detect the presence of ARF-1 in the Nef-MHC-I-AP-1 complex (Figure 4.4B). ARF-1 Q₇₁L co-precipitated readily, but both wild type and ARF-1 T₃₁N could also be detected. Of note, expression of ARF-1 mutants and BFA treatment had no significant effect on Nef co-precipitation with HLA-A2, confirming previous data (3,11) that Nef can bind to the cytoplasmic tail of HLA-A2 in the absence of AP-1.

To confirm these data, we also asked whether we could observe co-precipitation of AP-1, Nef and HLA-A2 when we immunoprecipitated ARF-1. For this study we examined complexes formed both in the absence and presence of Nef. Indeed, we were able to detect AP-1 co-precipitating with ARF-1 Q₇₁L, which was not too surprising given it's role in stabilizing AP-1 binding to cargo in the Golgi (34). In Nef-expressing cells we were also able to detect Nef co-precipitating with ARF-1 independent of the GTP-bound state of the ARF-1 molecule (Figure 4.5 lanes 4, 5, and 6), as previously reported (42). Additionally, in Nef expressing cells, there was an enhancement in the amount of AP-1 that co-precipitated with ARF-1 Q₇₁L (Figure 4.5, lane 6). Finally, we were also able to detect the presence of HLA-A2 co-precipitating with ARF-1 Q₇₁L in Nef expressing cells. These data suggest that ARF-1 Q₇₁L potentially stabilizes interactions amongst AP-1, Nef and MHC-I HLA-A2 and suggests that its mode of inhibition of MHC-I downmodulation is not disruption of complex formation but rather the inability of these complexes to dissociate once formed.

Discussion

HIV causes a persistent infection that evades eradication by anti-HIV CTLs. Viral persistence is mediated in part by the activity of HIV Nef protein, which disrupts antigen presentation by MHC-I to CTLs. In Nef expressing cells MHC-I accumulates in the *trans*-Golgi network and lysosomes and is degraded at a more rapid rate. AP-1 is required for Nef-dependent trafficking and AP-1 can be found in complexes with MHC-I and Nef in transformed T cell lines and in HIV-infected primary T lymphocytes (4).

In a previous report, we determined that the AP-1 μ 1 tyrosine binding pocket is important for AP-1 to be recruited to HLA-A2 by Nef (11). Here we have provide evidence that phosphorylation of AP-1 μ 1 is not required for Nef to downmodulate HLA-A2. In AP-2 μ 2, a phosphorylation site on a threonine at position 156 is required for endocytosis of transferrin (15) because the phosphorylation event at this site exposes the Yxx ϕ signal binding domain (14,19). Similarly, phosphorylation of the AP-1 μ 1 subunit dramatically increases AP-1's affinity for Yxx ϕ signals (18). However, we report that mutation of threonine residues in AP-1 μ 1 subunit that appear to reduce the efficiency of AP-1 trafficking of a Yxx ϕ signal (HLA-A2 YSQA₃₂₃V), are dispensable for Nef to downmodulate MHC-I. These data suggest that Nef is able to bypass the need for phosphorylation.

However, functional ARF-1 is necessary for Nef to recruit AP-1. This was best demonstrated by treatment with BFA, in which we observed a decrease in MHC-I downmodulation and a disruption of MHC-I-Nef-AP-1 complex formation. Moreover, the inhibitory effects of dominant active ARF-1 suggest that conversion of GTP bound ARF-1 to the GDP bound form is necessary for efficient disruption of antigen presentation. When ARF-1 Q₇₁L is expressed, ARF-1-GTP cannot be hydrolyzed into ARF-1-GDP resulting in a static clathrin coat (57,59); which compromises the cycles of AP-1 recruitment and dissociation from membranes. Collectively these data suggest that dissociation and recycling of coatomer subunits are important for efficient downmodulation of MHC-I. We propose that dissociation is needed for Nef to recruit β -COP, which we have shown is needed to efficiently target MHC-I for degradation. The

β -COP binding site on Nef is very closely opposed to the AP-1 binding site (5). Thus, it is likely that AP-1 needs to dissociate from the complex to allow β -COP to bind.

Work from a number of laboratories has supported a model in which the separate Nef-dependent trafficking pathways of MHC-I and CD4 ultimately converge into a common β -COP-dependent pathway necessary for lysosomal targeting (5,41,42). In this model, Nef promotes accelerated internalization of CD4 in an AP-2 dependent manner (60-63) and internalized CD4 is targeted into acidic compartments for accelerated degradation through ARF-1 and β -COP recruitment (5,41,42). Previous studies revealed that Nef-induced internalization of CD4 was not hindered by overexpression of any ARF-1 dominant inhibitory mutants (41) consistent with the fact that AP-2 does not require ARF-1 for its activity (64). Our results agree with previous data regarding CD4 downmodulation by Nef in the presence of ARF-1 mutants, yet strikingly Q₇₁L ARF-1 significantly affected downmodulation of MHC-I HLA-A2 by Nef. This difference supports our model that ARF-1 is required for recruitment of AP-1, which is needed for MHC-I, but not CD4 downmodulation.

A role for ARF-1 in Nef-dependent trafficking pathways has been explored previously with regard to adaptor protein stabilization on the *trans*-Golgi membrane (65), but our's is the first data implicating ARF-1 in AP-1 recruitment to MHC-I by Nef. Janvier *et al* utilized HeLa cells and either a CD8 α -Nef or a Nef-GFP fusion protein to conclude that ARF-1 is not needed for Nef to recruit AP-1 to the *trans*-Golgi. They reveal that Nef's dileucine motif is necessary for this phenotype. In contrast, downmodulation of MHC-I from the cell surface and AP-1 recruitment to MHC-I by Nef does not require the dileucine motif (D/ExxxLL) (4,11).

Functional ARF-6 is dispensable for Nef. Previous research suggests that ARF-6 is important for enhanced Nef-dependent endocytosis of MHC-I and that the dominant active Q₆₇L blocks internalization of MHC-I by Nef in HeLa cells (6). Another study explored overexpression of numerous ARF-6 mutants including the same dominant active ARF-6 molecule (ARF-6-Q₆₇L) and a double mutant that is GTP-bound and also cannot activate any downstream effectors (ARF-6-N48I;Q67L). Larsen *et al* determined that only ARF-6-Q₆₇L was unable to downmodulate MHC-I in the presence of Nef, thus suggesting ARF-6-Q₆₇L causes non-specific activation of downstream effectors leading

to an overall perturbation of membrane trafficking (66). Here we show in CEM SS CD4⁺ T cells, expression of ARF-6 dominant negative and dominant active molecules did not inhibit or enhance downmodulation of HLA-A2 by Nef.

In sum, we have further defined the mechanism by which Nef allows HIV-1 infected cells to evade the host immune response. Implicating ARF-1 in downmodulation of HLA-A2 by Nef provides further support for an AP-1-dependent model. Our prior studies have supported a model in which Nef binds to the hypophosphorylated cytoplasmic tail of HLA-A2 early in the secretory pathway (8) and upon arriving at the *trans*-Golgi network recruits AP-1 to the MHC-I cytoplasmic tail (3). Binding of AP-1 requires the tyrosine in the MHC-I cytoplasmic tail and the tyrosine binding pocket in AP-1 μ 1 (11,12,67). We now show putative phosphorylation sites in the AP-1 μ 1 subunit are not needed for this process but that normal function of the GTPase ARF-1 is necessary for efficient function of this complex. Our data thus far support a model in which AP-1 binding to Nef-MHC-I re-directs MHC-I from the plasma membrane into the endo-lysosomal network (4). AP-1 then dissociates allowing Nef to bind β -COP (68), which is recruited to target the MHC-I containing vesicles for degradation (5). These data help to elucidate the mechanism by which Nef downmodulates MHC-I therefore revealing further targets for inhibiting immune evasion.

Figure 4.1. Mutation of putative phosphorylation sites in AP-1 μ 1 does not affect Nef-induced downmodulation of wild type HLA-A2. (A) Measurement of HLA-A2 A₃₂₃V surface expression by flow cytometry. CEM-SS cells stably expressing HLA-A2 A₃₂₃V were transduced with Lentiviral vectors expressing GFP and the indicated shRNA.(shNC stands for negative control). The cells were subsequently treated with the indicated AP-1 retroviral constructs, which also expressed the marker gene placental alkaline phosphatase (PLAP). After 7 days of selection in puromycin, cells were stained with antibodies against PLAP and HLA-A2. GFP⁺PLAP⁺ cells were gated on for this analysis. The x-axes for HLA-A2 A₃₂₃V analyses extends from 100 to 100,000 fluorescence values. *Shaded curve*, shNC; *gray curve*, shAP-1 μ 1; *black curve*, shAP-1 μ 1 also expressing AP-1 μ R as marked. (B) Measurement of HLA-A2 surface expression by flow cytometry. CEM-SS cells stably expressing HLA-A2 were treated as in part A and then transduced with either negative control or Nef expressing adenovirus. 72 hours post adenoviral transduction, cells were stained with antibodies against PLAP and HLA-A2. GFP⁺PLAP⁺ cells were gated on for this analysis. *Shaded curve*, control adenoviral vector; *gray curve*, adenoviral vector expressing Nef (adeno-Nef) without AP-1 μ R; *black curve*, adeno-Nef with AP-1 μ R as marked. (C) Quantitation of part (A). The median fluorescence normalized to shNC is shown \pm standard deviation (sd) $n=2-7$. Paired T tests were performed comparing populations as indicated by black bars. Comparing shNC to shAP-1, shAP-1 + TBPM AP-1 μ R, or shAP-1 + T₁₅₄A AP-1 μ R revealed p -values of 0.0001, 0.0001, and 0.046 respectively. Comparing shAP1 + wild type AP-1 μ R to shAP-1 + TBPM AP-1 μ R, shAP-1 + T₁₅₄A AP-1 μ R, or shAP-1 + T_{152/4}A AP-1 μ R revealed p -values of 0.004, 0.001, and 0.85 respectively. * denotes a p -value of <0.05 , ** denotes a p -value of <0.01 , and *** denotes a p -value of <0.001 . (D) Quantitation of part (B). The median fluorescence normalized to shNC is shown \pm standard deviation (sd) $n=3-6$. Paired T tests were performed comparing populations as indicated by black bars. p -values. Comparing shNC to shAP-1 or shAP-1 + TBPM AP-1 μ R revealed p -values of 0.009 and 0.01 respectively. Comparing shAP1 to shAP-1 + wild type AP-1 μ R revealed a p -value of 0.005. Comparing shAP1 + wild type AP-1 μ R to shAP-1 + TBPM AP-1 μ R revealed a p -value of 0.02.

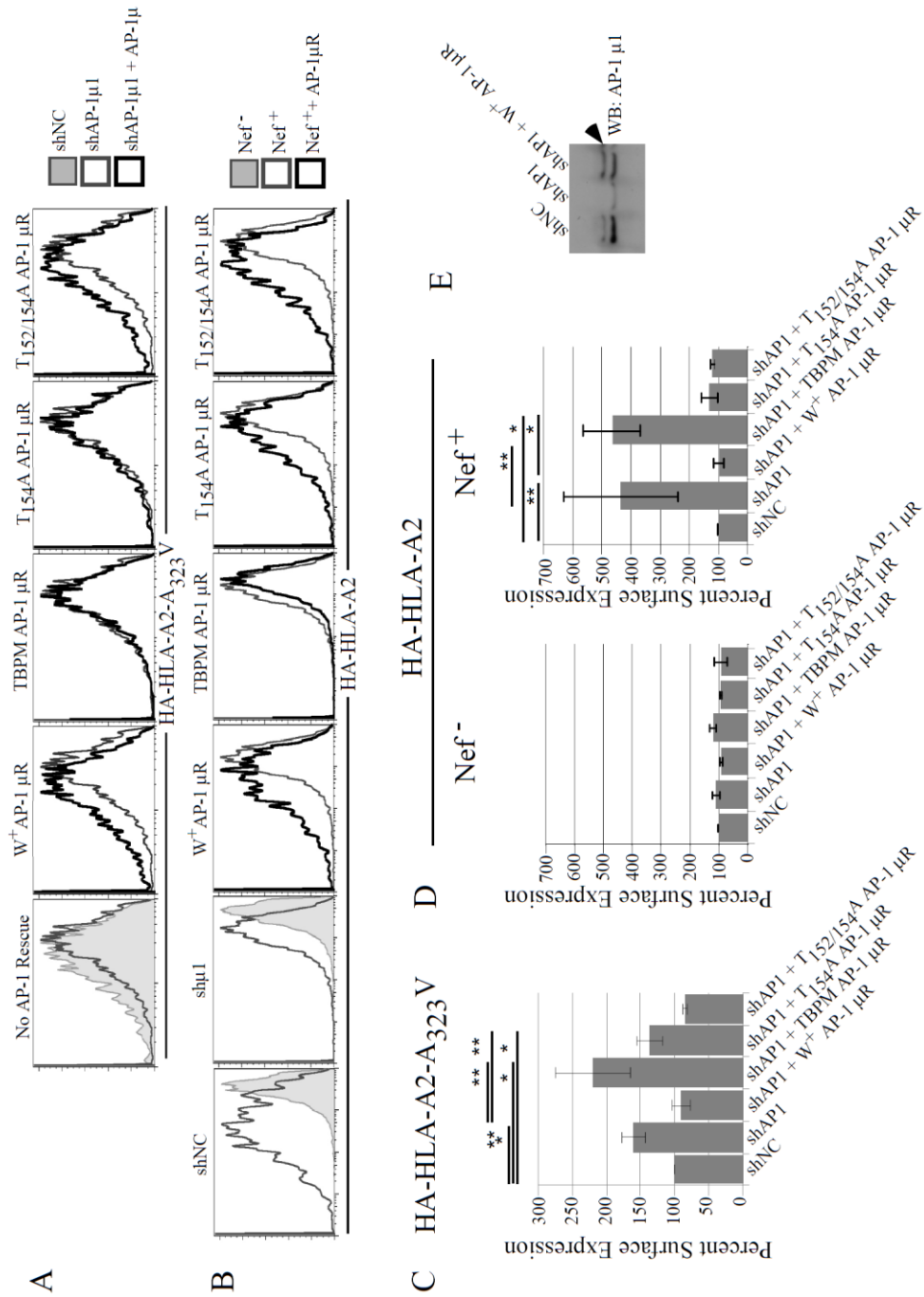


Figure 4.1. Mutation of putative phosphorylation sites in AP-1 μ 1 does not affect Nef-induced downmodulation of wild type HLA-A2

Figure 4.2. ARF-1 activity is required for Nef-induced downmodulation of HLA-A2. (A) Flow cytometric analysis of HLA-A2, HLA-A2 A₃₂₃V, and CD4 surface expression. CEM-SS HLA-A2 or HLA-A2 A₃₂₃V cells were first transduced with either control or Nef-expressing adenoviral vectors. Then, cells were either transduced with a bicistronic murine retroviral vector expressing GFP and either wild type or mutant ARF-1 or treated with BFA as noted.. For ARF-1 samples, similar GFP⁺ populations based on FL-1 mean were analyzed for HLA-A2, HLA-A2 A₃₂₃V or CD4 as marked. *Dark gray shaded curve*, vector alone; *Light gray shaded curve*, adeno-Nef; *black curve*, adeno-Nef plus ARF-1 as indicated. (B) Quantitation of part (A). The median fluorescence normalized to wild type ARF-1 is shown \pm standard deviation (sd) $n=3-13$. Paired T tests were performed comparing populations as indicated by black bars. * denotes a p -value of <0.05 , ** denotes a p -value of <0.01 , and *** denotes a p -value of <0.001 . Comparing HLA-A2 Nef⁺ wild type to HLA-A2 Nef⁺ Q71L ARF-1 in the presence of Nef yielded a p -value of 0.002. Comparing HLA-A2-A₃₂₃V Nef⁺ wild type ARF-1 to HLA-A2-A₃₂₃V Nef⁺ Q71L ARF-1 in the presence of Nef yielded a p -value of 0.008. (C) Quantitation of brefeldin A treatment from part (A). The median fluorescence normalized to BFA⁻ is shown \pm standard deviation (sd) $n=3-13$. Paired T tests were performed comparing populations as indicated by black bars. Comparing A2 + Nef BFA⁻ to A2 + Nef BFA⁺ yielded a p -value of 2×10^{-5} . Comparing A₃₂₃V + Nef BFA⁻ to A₃₂₃V + Nef BFA⁺ yielded a p -value of 8×10^{-8} .

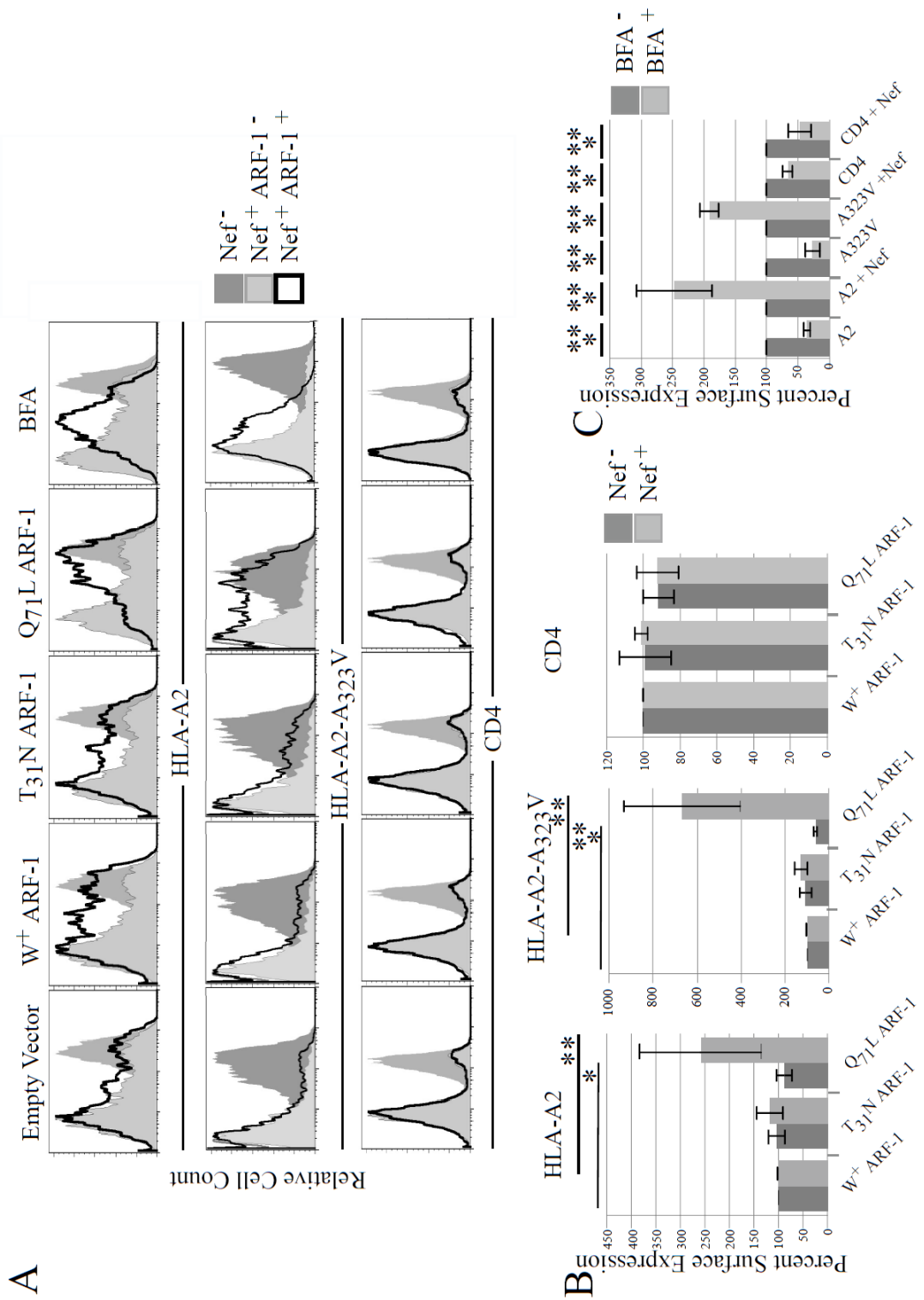


Figure 4.2. ARF-1 activity is required for Nef-induced downmodulation of HLA-A2.

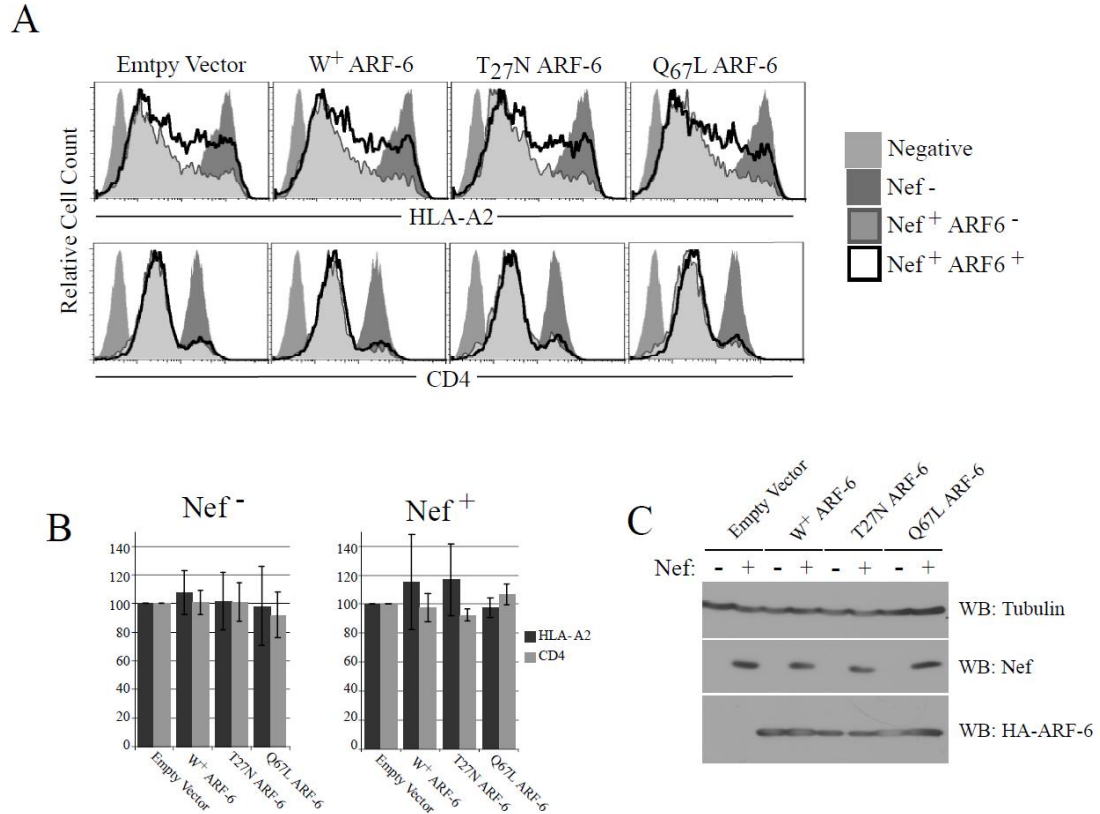


Figure 4.3. ARF-6 is uninvolved in HLA-A2 and CD4 downmodulation by Nef.

(A) Analysis of HLA-A2 or CD4 surface expression in the presence of Nef and ARF-6 mutants. CEM-SS HLA-A2 cells were first transduced with control or Nef-expressing adenovirus. Then, cells were transduced with a bicistronic murine retroviral vector expressing GFP and either wild type or mutant ARF-6. Similar GFP⁺ populations based on FL-1 mean were analyzed for HLA-A2 or CD4 as indicated. *Medium gray shaded curve*, isotype control stain; *Dark gray shaded curve*, control vector; *Light gray shaded curve with dark outline*, adeno-Nef; *black curve*, adeno-Nef plus ARF-6 as indicated. (B) Quantitation of part (A). The median fluorescence normalized to wild type ARF-1 is shown \pm standard deviation (sd) $n=3$. (C) Western blot of ARF-6 and Nef expression.

Figure 4.4. Dominant active ARF-1 stabilizes AP-1 binding to the Nef-MHC-I complex. (A) MHC-I HLA-A2 A₃₂₃V was immunoprecipitated from CEM-SS cells transduced with a bicistronic murine retroviral vector expressing GFP and ARF-1 as indicated. Based on GFP expression 50-70% of the cells were transduced. AP-1 in complex with HLA-A2 A₃₂₃V was detected by western blot analysis. (*n*=4) (B) MHC-I HLA-A2 was immunoprecipitated from cells transduced with adeno-Nef and a bicistronic murine retroviral vector expressing GFP and either wild type or mutant ARF-1. Based on GFP expression 50-70% of the cells were transduced. Proteins in complex with HLA-A2 were detected through western blot as indicated. (*n*=4)

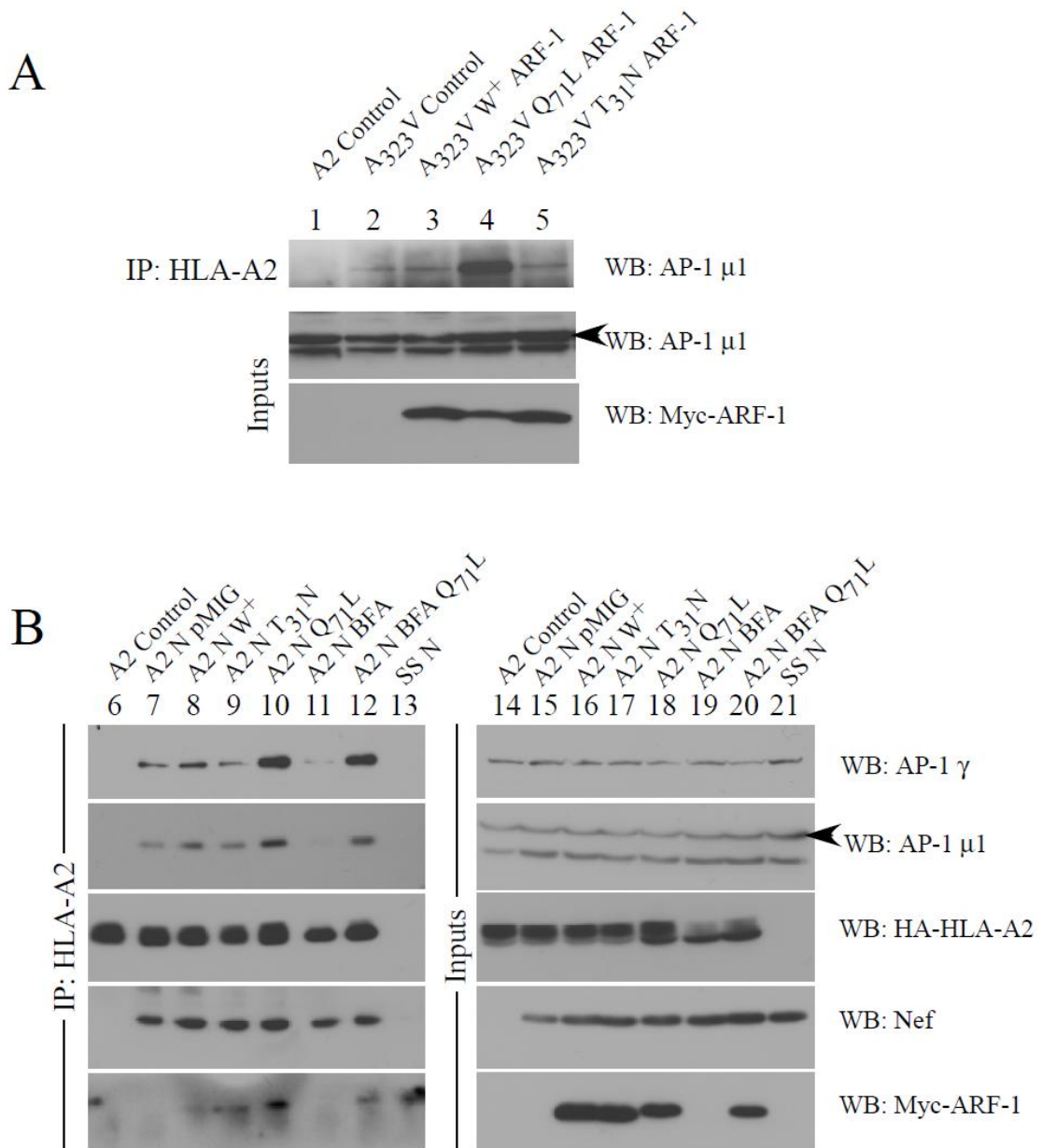


Figure 4.4. Dominant active ARF-1 stabilizes AP-1 binding to the Nef-MHC-I complex.

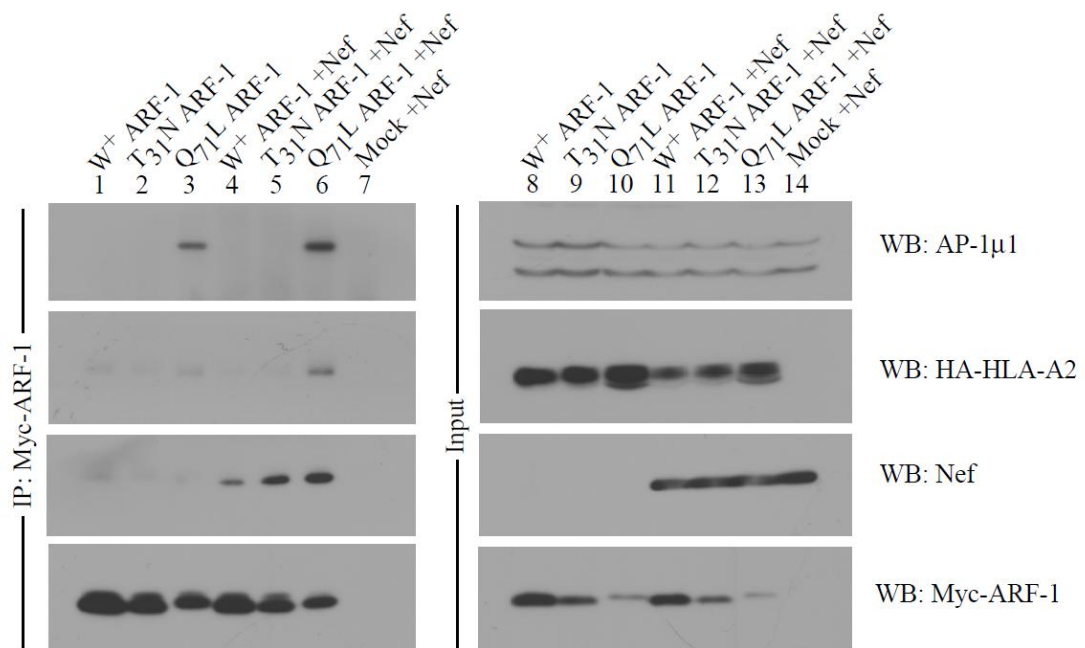


Figure 4.5. Nef stabilizes the interaction between ARF-1 and AP-1.

Myc-tagged ARF-1 was immunoprecipitated from cells, treated as in Figure 4.4 part B. Based on GFP expression 50-70% of the cells were transduced. Proteins in complex with ARF-1 were detected through western blot as indicated. ($n=2$)

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CHAPTER V

Discussion

Summary of Results

HIV Nef has been shown to aid in disease progression and viral fitness by helping the virus to evade adaptive and innate immune recognition through selectively downmodulating antigen presenting MHC-I molecules. Nef binds to the cytoplasmic tail of the MHC-I allotype HLA-A2 early in the secretory pathway and upon arrival at the *trans*-Golgi network, recruits the clathrin adaptor protein AP-1. This three-way complex is required to retain MHC-I in the *trans*-Golgi to endosome loop.

In Chapter II, through extensive mutational analysis, we were able to successfully map domains in HLA-A2, Nef, and AP-1 that are required for the three-way complex formation. We found that Y₃₂₀ was the only amino acid in the MHC-I cytoplasmic tail absolutely required for Nef binding. In contrast, AP-1 recruitment to the Nef-MHC-I complex required Y₃₂₀, A₃₂₄, and D₃₂₇ in the MHC-I cytoplasmic tail. Each of these amino acids is differentially expressed between various HLA molecules. MHC-I molecules, HLA-A and HLA-B, are downmodulated by Nef and contain these three key amino acids. However, natural killer cell inhibiting MHC-I molecules, HLA-C and HLA-E, each contain at least one mutation at these key AP-1 recognition amino acids and are not downmodulated by Nef. Thus, Nef is able to limit recognition by both the adaptive and innate immune response.

The acidic cluster and polyproline repeat in Nef were previously shown to be dispensable for AP-1 recruitment (1). Here through more stringent techniques, we revealed that each domain functions to stabilize the interaction between AP-1 and MHC-I. Additionally, we demonstrated that the natural tyrosine-binding pocket (TBP) in AP-1 was necessary for Nef-induced MHC-I downmodulation and for AP-1 to bind HLA-A2.

Creation of a Yxx ϕ canonical AP-1 signal (Y₃₂₀SQV₃₂₃) allowed an interaction between HLA-A2 and AP-1 in the absence of Nef. In sum, these data support the model that multiple Nef domains work together to allow Y₃₂₀ in the MHC-I cytoplasmic tail to behave as a Yxx ϕ AP-1 sorting signal.

Since this paper was published in 2008 (2), numerous manuscripts have been able to confirm that various Nef domains are important for AP-1 stabilization in the Nef-MHC-I complex (3-5). In contrast to published PACS-1 literature, Baugh *et al* was able to reveal that the acidic cluster in Nef does not readily interact with the PACS-1 furin binding region (4). In fact, mutating three of the four glutamates in this cluster only decreased Nef's effects on MHC-I downmodulation by about 50%, suggesting that the function of this domain is to stabilize the Nef-MHC-I-AP-1 complex rather than recruit a separate sorting protein. Furthermore, Luben *et al* revealed that knocking down PACS-1 and PACS-2 expression did not actually affect the cellular localization of furin or inhibit MHC-I downmodulation by Nef, thus suggesting PACS-1 and PACS-2 do not sort proteins that contain acidic clusters (6). In addition, two papers from the Guatelli group have confirmed Nef's acidic cluster and polyproline repeat stimulates AP-1 μ 1 to directly interact with the cryptic YxxA signal in the HLA-A2 cytoplasmic tail (3,5). In addition, it was confirmed that AP-1's TBP was responsible for the direct interaction with the cryptic YxxA signal in HLA-A2 (5).

Chapter III discusses the convergence of two different pathways by which Nef affects MHC-I and CD4 surface expression. As mentioned previously, MHC-I is rerouted into the endosomal network and subsequently transported to late endosomes and finally lysosomes for degradation. In contrast, CD4 is endocytosed into the endosomal network, where it is found to co-localize with MHC-I in Rab7⁺ late endosomes. We determine that the COP-I coatomer β -COP is the common factor utilized by Nef to transport MHC-I and CD4 into lysosomal compartments for degradation. Finally, we were able to identify two domains in Nef that were necessary for β -COP binding; each domain was responsible for degrading either MHC-I or CD4. These results explain how each domain could have been utilized differently in various experimental setups, thus clearing up discrepancies found in domain usage in previously published literature (7-10). Explicitly, mutating an RXR motif in the N-terminal α helix halved β -COP binding and

inhibited MHC-I degradation. In addition, mutating a diacidic motif (EE_{155, 156}) in the C-terminal flexible loop of Nef also reduced β -COP binding by one half and inhibited CD4 degradation, thus confirming previous data that the diacidic motif (EE_{155, 156}) was required for CD4 transport into acidic compartments (9). Overall, these data support a model in which both MHC-I and CD4 are ultimately targeted to lysosomes in Nef-expressing cells by a final β -COP-dependent pathway.

In Chapter IV, we explored MHC-I downmodulation by Nef in the presence of AP-1 phosphorylation mutants and mutants of the AP-1 recruitment protein ARF-1. Using an HLA-A2 molecule mentioned previously (Y₃₂₀SQV₃₂₃), we were able to explore AP-1 phosphorylation biology in the absence of Nef. Indeed, an AP-1 phosphorylation mutant (T₁₅₄A) was less able to maintain HLA-A2-Y₃₂₀SQV₃₂₃ inside the cell, thus increasing its surface expression by about 50%. In contrast, Nef was able to utilize the mutant form of AP-1 to downmodulate HLA-A2, suggesting that Nef does not require phosphorylation of AP-1 for Nef's function.

In addition, we determined that Nef required the functional recruitment of the AP-1 scaffold protein ARF-1. We inhibited ARF-1 by either treating cells with the GEF inhibitor brefeldin A or by overexpressing dominant inhibitory molecules. Brefeldin A treatment inhibited MHC-I downmodulation and AP-1 recruitment to the Nef-MHC-I complex. Brefeldin A treatment did not disturb Nef binding to HLA-A2, confirming that Nef directly interacts with the cytoplasmic tail of HLA-A2 in the absence of AP-1 (11). In addition, overexpressing the dominant active ARF-1 molecule (Q₇₁L) was able to reverse brefeldin A inhibition of AP-1 recruitment. This reveals that despite Golgi dissociation caused by brefeldin A, the loss of AP-1 recruitment was directly related to the inhibition of ARF-1 rather than a loss of Golgi structure. These effects were ARF-1 specific because dominant inhibitory ARF-6 molecules did not affect downmodulation of HLA-A2 by Nef. Finally, we revealed that Nef stabilizes the interactions between ARF-1 and AP-1. These data further solidify the AP-1 dependent model of Nef-induced downmodulation of MHC-I by implicating the AP-1 regulatory protein ARF-1.

It was previously shown that Nef is in fact able to stabilize adaptor protein complexes on the Golgi membrane (12,13). The dileucine motif in Nef was shown to specifically recruit AP-1 to Golgi membranes in a brefeldin A-insensitive manner

(12,13). In contrast, MHC-I downmodulation has been shown to be independent of Nef's dileucine motif (1,14,15). Thus, this evidence suggests that Nef requires ARF-1 to recruit AP-1 to a cryptic Yxx ϕ signal rather than its own dileucine motif.

Based on the results presented and discussed in this dissertation, a comprehensive model of Nef downmodulating MHC-I and CD4 is proposed in Figure 5.1. Nef's hydrophobic pocket (WL_{57,58}) binds to the cytoplasmic tail of CD4 at the plasma membrane and Nef's C-terminal flexible loop forms a bridge to the endocytic adaptor protein AP-2. CD4 is then endocytosed into the endosomal network. In contrast, MHC-I is bound early in the secretory pathway where Nef turns a cryptic signal into a Yxx ϕ sorting motif in the cytoplasmic tail of MHC-I. Upon reaching the *trans*-Golgi network, ARF-1 helps to recruit AP-1's tyrosine binding pocket to the MHC-I cytoplasmic tail. Domains in MHC-I, Nef, and AP-1 stabilize the complex and allow MHC-I to be transported into the endosomal network. Here, the separate pathways that Nef utilizes to downmodulate CD4 and MHC-I converge in Rab7⁺ late endosomal compartments and β -COP is recruited to aid in the degradation of each molecule. Recent evidence suggests that endosome to *trans*-Golgi recovery and accumulation is dependent upon the retromer complex, thus retromer could be responsible for recovering MHC-I from late endosomes (16,17).

Future Directions

Several key questions remain regarding the mechanism by which Nef downmodulates and degrades MHC-I.

1. What is nature of ARF-1 and β -COP interactions with MHC-I?
2. Are any AP-1 kinases or phosphatases involved in the cyclical activation and recruitment of AP-1 by Nef?
3. Is reversal of downmodulation caused in each previously discussed experiment physiologically relevant?
4. Ultimately, how can we inhibit HIV immune evasion in infected individuals?

Each of these major points will be explored below.

What is nature of ARF-1 and β -COP interactions with MHC-I? As of yet, the sequence of events regarding adaptor protein recruitment to the Nef-MHC-I complex remains elusive. It has been suggested that Nef binds to hypophosphorylated MHC-I early in the secretory pathway (18). Upon reaching the *trans*-Golgi network, AP-1 is recruited in an ARF-1-dependent manner. It would be useful to determine if Nef is directly interacting with and recruiting ARF-1 to the *trans*-Golgi or if the recruitment is indirect. Nef is able to localize the ARF-6 GEF, ARNO, to the plasma membrane and subsequently enhance GTP loading into the ARF-6 molecule (19). Therefore, Nef could be recruiting an ARF-1 GEF to activate and indirectly recruit ARF-1.

Defining a domain in Nef that is required for ARF-1 recruitment would aid in determining the overall mechanism used by Nef to recruit AP-1 to MHC-I. Thus far, the C-terminal flexible loop of Nef has not been important for Nef to affect MHC-I; additionally a diacidic domain in Nef (EE_{155, 156}) is reported to interact with ARF-1 to localize CD4 to acidic compartments (9). In Chapter III, we revealed that there are two β -COP binding sites on Nef, each being used to degrade either MHC-I or CD4. Because it is located in the C-terminal flexible loop, it would be surprising for Nef's diacidic motif (EE_{155, 156}) to be responsible for ARF-1 recruitment to the Nef-MHC-I complex. This evidence suggests that Nef contains another ARF-1 recruitment domain for Nef to downmodulate MHC-I. It would be interesting to utilize the A2/Nef fusion protein to determine if the acidic cluster (E₆₂₋₆₅) or the polyproline repeat (P_{72/75/78}) in Nef are important domains for full AP-1 stabilization because they bind to ARF-1.

In this dissertation, we determined that ARF-1 is involved in AP-1 recruitment to the Nef-MHC-I complex. In addition, we demonstrated that β -COP is a factor in the degradation of MHC-I. ARF-1 was shown to be important for β -COP recruitment to membranes (20,21) and for CD4 localization to acidic compartments by Nef (9). It has yet to be determined if ARF-1 is involved in β -COP recruitment to degrade MHC-I. In addition, the nature by which β -COP is involved in degrading MHC-I has yet to be defined, as the Nef-MHC-I- β -COP complex remains elusive. It is possible that in order to degrade MHC-I, ARF-1 could recruit β -COP, rather than Nef, after AP-1 uncoats from the clathrin coat. In our model, we cannot definitively say that β -COP is directly interacting with each domain of Nef. By immunoprecipitating from a lysate, we cannot

rule out the possibility that previously formed complexes could contain another factor that acts as a bridge between β -COP and Nef; ARF-1 could be a likely candidate. Additionally, because β -COP is less traditionally thought of as part of the degradative pathway, it would be interesting to determine if β -COP localization to acidic compartments is enhanced by Nef expression.

As mentioned previously, Nef requires two domains to recruit β -COP for MHC-I and CD4 degradation. It would be interesting to determine the reason behind differential domain usage in Nef to affect different molecules. It is possible that upon binding to the cytoplasmic tail of each cargo protein, Nef assumes a dramatically different conformation that exposes either the C-terminal flexible loop or core domains. Significant understanding of Nef's functions and domain usage would be gained through conformational analysis via NMR or by solving the crystal structure of Nef in complex with various cytoplasmic tails.

Macrophages are professional antigen presenting cells in the immune system. They phagocytose samples from their environment and digest exogenous proteins into smaller peptide fragments that are presented by MHC-II molecules. These MHC-II-peptide complexes are then detected by antigen specific CD4⁺ or CD8⁺ T cells to initiate an immune response. Nef has been shown to decrease the surface level of MHC-II molecules in order to decrease anti-HIV immune activation (22). In addition, Nef selectively downmodulates the mature MHC-Class II molecules, while leaving the immature versions on the plasma membrane. Nef preferentially binds hypophosphorylated MHC-I (18) and it would be fascinating to determine if phosphorylation on the cytoplasmic tail of MHC-II affects its sensitivity to Nef. Additionally, phagocytosis in macrophages has been shown to involve AP-1 and ARF-1 at the plasma membrane (23). The mechanism appears to be brefeldin A insensitive and clathrin-independent, thus suggesting a different mechanism of action by AP-1 and ARF-1 in different cell types. It would be interesting to determine if Nef is recruiting excessive AP-1 or ARF-1 to cause accelerated phagocytosis at the plasma membrane.

Are any AP-1 kinases or phosphatases involved in the cyclical activation and recruitment of AP-1 by Nef? Nef has numerous domains that have been shown to interact with multitudes of kinases. The N-terminal α helix and polyproline repeat

(P_{72/75/78}) have been shown to interact with Src family kinases such as Hck and Lck (24-26). A di-arginine repeat (RR_{105,106}) has been shown to interact with PAK1 and PAK2 (26,27). The C-terminal flexible loop has been shown to interact with PI-3-Kinase (28). None of these kinases have been implicated in the cyclic phosphorylation of AP-1; thus it is possible that an AP-1-specific kinase that remains thus far unidentified is responsible for cyclic AP-1 recruitment or dissociation by Nef.

The cyclical activation and uncoating of AP-1 to and from vesicles involves the phosphorylation and dephosphorylation of AP-1 subunits. It would be interesting to determine how Nef affects the recruitment, activation, and dissociation of AP-1 coated vesicles in relation to MHC-I downmodulation. Thus far, the G cyclin-associated kinase (GAK) has been shown to be recruited into clathrin coated pits (29-31) and to phosphorylate the μ subunit of AP-1 and AP-2 (32), causing a relaxed conformation that exposes the tyrosine binding pocket in each (33). It has also been suggested that GAK could be involved in uncoating AP-1 from endosomes by phosphorylating the β subunit of AP-1 (29,31,32). Knocking down the expression of GAK causes an inhibition of localization of AP-1, AP-2, clathrin, and GGAs to membranes (34). The involvement of GAK in either CD4 internalization or recruitment of AP-1 to the Nef-MHC-I complex remains to be explored.

AP-1 μ 1 has been shown to be phosphorylated in clathrin coated vesicles and β 1 has been shown to be phosphorylated in the cytosol (33,35), suggesting that their localization is phosphorylation-dependent. The phosphatase PP2A has been shown to dephosphorylate either the μ or β 1 subunit appropriately depending on their stage of vesicle formation. AP-1 released from clathrin coated vesicles by PP2A was unable to bind sorting signals (33). By utilizing the PP2A inhibitor okadaic acid, it would be interesting to determine if Nef affects the recruitment or activity of PP2A.

Is reversal of downmodulation caused in each previously discussed experiment physiologically relevant? The actual significance of the research presented thus far should be further confirmed through experiments performed with full-length HIV in primary cells. Experimental procedures utilized in this dissertation were generally performed in immortalized CD4⁺ lymphoblastoid cell lines with Nef expressed from a replication deficient Adenovirus and mutated cellular proteins expressed from a

bicistronic retrovirus. Revealing a requirement for β -COP and ARF-1 in primary CD4⁺ T cells would confirm the mechanism we have proposed thus far. Now that we have revealed that specific domains in Nef are important for β -COP recruitment, we could mutate these domains in *nef* in the HIV genome to explore the downmodulation and degradation of MHC-I and CD4 in primary CD4⁺ T cells. In addition, disproving MHC-I downmodulation by an ARF-6 dependent pathway in primary CD4⁺ T cells would further confirm that Nef functions primarily by reducing the transport of MHC-I from the *trans*-Golgi to the plasma membrane rather than enhancing its endocytosis.

Our lab has developed an HIV construct that encodes a GFP reporter and overexpresses any non-HIV protein of interest. This tool is being used to express the AP-1 tyrosine binding-pocket mutant (FD_{172/174}) from Chapter II and the ARF-1 or ARF-6 dominant inhibitory molecules from Chapter IV. In future experiments, these viruses will allow us to explore the involvement of AP-1, ARF-1 and ARF-6 in MHC-I downmodulation by Nef in HIV-infected primary CD4⁺ T cells.

Furthermore, these HIV constructs could be utilized to determine if the reversal of MHC-I downmodulation by mutating AP-1 or ARF-1 is functionally significant. Primary CD4⁺ T cells infected with these HIV constructs could be incubated with anti-HIV CTL clones in a co-culture assay to determine if the reversal of downmodulation is enough to render these HIV infected cells sensitive to CTL lysis.

Ultimately, how can we inhibit HIV immune evasion in infected individuals?

Because Nef is required for disease progression and fitness, inhibiting Nef's effects on immune evasion may limit HIV disease. A compound or small molecule designed to specifically inhibit the Nef-MHC-I interaction would be expected to rescue viral antigen presentation to CTLs, thus diminishing the evolution of drug resistant viruses and allowing the infected individual's immune system to maintain low-level viremia without the toxic side-effects of anti-retroviral therapies.

Finally, could inhibiting the formation of the Nef-MHC-I complex allow for host control of HIV infection? HIV lays dormant in latent cellular reservoirs and causes cyclical blips of viremia even while a patient is on anti-retroviral therapies. Allowing the immune system to recognize and clear newly infected cells may allow for a cyclical

resurgence of viremia but not eventual immune system collapse, opportunistic infections, and death.

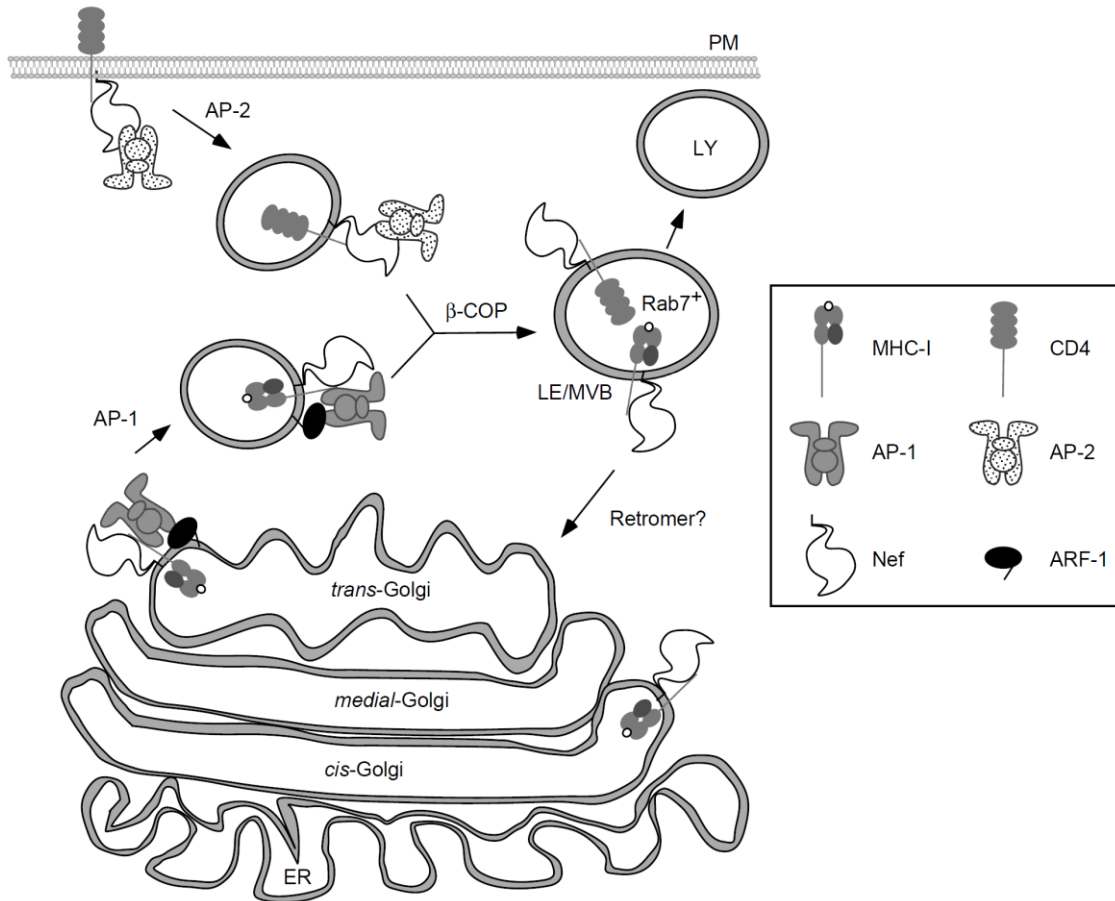


Figure 5.1. Nef downmodulates MHC-I and CD4 via two separate pathways which converge in a COP-I coatmer β -COP-dependent degradative pathway.

Nef binds to the cytoplasmic tail of CD4 at the plasma membrane (PM) and recruits AP-2 to promote endocytosis. CD4 is internalized and is localized to Rab7⁺ late endosomes (LE/MVB). In contrast, Nef binds to the cytoplasmic tail of MHC-I early in the secretory pathway and upon reaching the *trans*-Golgi recruits AP-1 in an ARF-1-dependent manner. MHC-I is also localized to Rab7⁺ late endosomes. Upon MHC-I and CD4 reaching a common late endosomal compartment, Nef recruits β -COP to promote degradation of each molecule in lysosomes (LY).

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