HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail

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To avoid immune recognition by cytotoxic T lymphocytes (CTLs), human immunodeficiency virus (HIV)-1 Nef disrupts the transport of major histocompatibility complex class I molecules (MHC-I) to the cell surface in HIV-infected T cells. However, the mechanism by which Nef does this is unknown. We report that Nef disrupts MHC-I trafficking by rerouting newly synthesized MHC-I from the trans-Golgi network (TGN) to lysosomal compartments for degradation. The ability of Nef to target MHC-I from the TGN to lysosomes is dependent on expression of the μ1 subunit of adaptor protein (AP) AP-1A, a cellular protein complex implicated in TGN to endosomal pathways. We demonstrate that in HIV-infected primary T cells, Nef promotes a physical interaction between endogenous AP-1 and MHC-I. Moreover, we present data that this interaction uses a novel AP-1 binding site that requires amino acids in the MHC-I cytoplasmic tail. In sum, our evidence suggests that binding of AP-1 to the Nef–MHC-I complex is an important step required for inhibition of antigen presentation by HIV.

Introduction

The human immunodeficiency virus (HIV) establishes a chronic infection in T lymphocytes by evading the host immune response. To prevent recognition and killing by host cytotoxic T lymphocytes (CTLs), HIV reduces the cell surface expression of major histocompatibility complex class I molecules (MHC-I) (Schwartz et al., 1996), which are needed to present antigenic peptides to CTLs. The HIV Nef protein is required for the reduced MHC-I cell surface expression (Schwartz et al., 1996) and to thereby protect HIV-infected primary T cells from anti-HIV CTLs (Collins et al., 1998).

HIV-1 Nef is a 27–34-kD multifunctional protein that has no apparent enzymatic activity and is thought to function by acting as an adaptor protein (AP). Consistent with this, Nef binds to cytoplasmic tail domains (Harris and Neil, 1994; Greenway et al., 1995; Grzesiek et al., 1996; Rossi et al., 1996; Schaefer et al., 2000; Williams et al., 2002), and contains a number of potential protein–protein interaction domains that are required either for MHC-I or CD4 downmodulation. An NH2-terminal α-helical domain, an acidic domain and a polyproline repeat are all required for MHC-I downmodulation (Greenberg et al., 1998b; Mangasarian et al., 1999). In contrast, dileucine and diacidic motifs within Nef are necessary for removal of cell surface CD4 (Aiken et al., 1994; Greenberg et al., 1998b).

Thus far, Nef has been shown to downmodulate all MHC-I HLA-A and HLA-B allotypes. However, Nef does not downmodulate MHC-I HLA-C and HLA-E (Le Gall et al., 1998; Cohen et al., 1999). This selective downmodulation results from amino acid sequence variation in the Nef binding domain within the cytoplasmic tails of these molecules (Williams et al., 2002). Because HLA-C and HLA-E are known to inhibit natural killer cell lysis, it has been proposed that maintenance of their cell surface expression may allow survival of the infected cell (Le Gall et al., 1998; Cohen et al., 1999).

The current model for how Nef affects CD4 cell surface expression is that Nef binds to the CD4 cytoplasmic tail and links it to cellular trafficking proteins that accelerate its endocytosis. Indeed, Nef is known to interact with a number of proteins that regulate intracellular trafficking. For example, Nef interacts with the heterotetrameric clathrin APs (AP-1, AP-2, and/or AP-3; Bresnahan et al., 1998; Greenberg et al., 1998a; Le Gall et al., 1998; Piguet et al., 1998; Craig et al., 2000; Erdtmann et al., 2000; Janvier et al., 2003a,b), through the same dileucine motif that is needed for CD4 downmodulation (Bresnahan et al., 1998; Greenberg et al., 1998a; Craig et al., 2000; Janvier et al., 2003a,b).

The adaptin complexes (Robinson and Bonifacino, 2001; Traub, 2003; Robinson, 2004) are each composed of four subunits; two large subunits (β1, β2, β3 plus γ, α, or δ), medium...
subunit (μ1A, μ1B, μ2, μ3) and one small subunit (σ1, σ2, σ3) for AP-1 (A or B), AP-2 and AP-3, respectively. There is evidence that all of these complexes sort proteins by binding recognition sequences in their cytoplasmic tails and linking them to clathrin. AP-1 is localized to the TGN and endosomes. Thus, it is thought to be important for trafficking between these compartments (Doray et al., 2002; Waguri et al., 2003), and for eventual sorting of some proteins (e.g., lysosomal hydrolases) to the lysosomes. AP-3 is localized to endosomes (Peden et al., 2004) and is needed for proper targeting of other proteins (e.g., lysosome-associated membrane protein [LAMP-1]) to lysosomes. Finally, AP-2 is localized to the cell surface and plays a role in endocytosis (Traub, 2003). Thus, one model is that Nef-dependent CD4 endocytosis occurs via recruitment of AP-2 through Nef’s dileucine motif. Nef-dependent CD4 endocytosis may additionally require interaction with a subunit of the vacuolar ATPase (Lu et al., 1998), which may indirectly promote an association of Nef with AP-2 (Geyer et al., 2002). However, the mechanistic details of how interactions of Nef with various adaptin molecules accelerate CD4 endocytosis and degradation are not well understood.

There is increasing evidence that Nef disrupts MHC-I cell surface expression by a different mechanism than it uses to downmodulate CD4. The diacidic motif needed for Nef to interact with the vacuolar ATPase to promote CD4 down-regulation is not required for Nef to disrupt MHC-I trafficking (Greenberg et al., 1998b; Lu et al., 1998). In addition, the dileucine motif within Nef that is required for Nef to directly bind adaptin complexes is also dispensable (Greenberg et al., 1998b; Mangasarian et al., 1999). Moreover, in HIV-infected T lymphocytes and astrocytic cells, the primary effect of Nef on MHC-I is to disrupt its transport to the cell surface rather than to promote its endocytosis (Swann et al., 2001; Kasper and Collins, 2003). The molecular mechanism underlying this effect of Nef is unknown.

To determine how Nef disrupts the transport of MHC-I to the cell surface, we used biochemical and cell biological approaches to examine MHC-I trafficking in Nef-expressing T cells. We found that Nef redirected MHC-I from the TGN to lysosomes. Moreover, we demonstrated that the AP, AP-1, which is required for proper sorting of lysosomal hydrolases from the TGN to lysosomes, was required for Nef to disrupt MHC-I trafficking. Finally, in HIV-infected primary T cells, we found that Nef stabilized an interaction between MHC-I and AP-1. This interaction used a novel domain that required sequences from the NH2-terminal α helix of Nef and from the MHC-I cytoplasmic tail.

Results

To perform large scale biochemical experiments to examine the effects of Nef on MHC-I trafficking in T cells, we developed a system that permitted transient, uniform, expression of Nef in T cells. This was accomplished by using a replication-defective adenoviral vector expressing HIV-1 Nef, which disrupts MHC-I transport in a manner that is indistinguishable from HIV (Kasper and Collins, 2003). In addition, to specifically detect an allotype of MHC-I that is responsive to Nef, we attached an HA tag to the NH2-terminal extracellular domain of HLA-A2. Based on a number of biochemical measurements, the tagged molecule was folded properly, matured through the secretory pathway normally, and was equally responsive to HIV-1 Nef (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407031/DC1).

Trafficking of MHC-I in Nef-expressing T cells

It has been previously shown that Nef does not affect the transport of MHC-I into the medial Golgi apparatus (Schwartz et al., 1996), but does inhibit its transport to the cell surface (Kasper and Collins, 2003). To further define where in the secretory pathway Nef exerts its effects, we asked whether MHC-I was transported normally into the TGN. This was accomplished by monitoring the rate at which HLA-A2 acquired sialic acid residues in this compartment, which can be measured by testing sensitivity to neuraminidase digestion. We detected sialylation of HLA-A2 as a slight increase in HLA-A2 molecular weight (Fig. 1 A, lanes 4 and 7) that was eliminated by neuraminidase treatment (Fig. 1 A, lanes 5 and 8). Based on these data (quantified in Fig. 1 B), Nef does not delay the transport of HLA-A2 into the TGN. As a control, we also confirmed the previously published observation that Nef does not affect transit into the medial Golgi (Schwartz et al., 1996), as assessed by sensitivity to endoglycosidase H (endo H) digestion (Fig. 1, A and B).

HIV-1 Nef causes HLA-A2 to be degraded in lysosomal compartments

There is consensus that Nef causes the accumulation of MHC-I in the TGN of multiple non-T cell lines (Schwartz et al., 1996; Greenberg et al., 1998b; Le Gall et al., 1998; Piguet et al., 2000; Swann et al., 2001; Blagoveshchenskaya et al., 2002). However, it is controversial as to whether MHC-I is ultimately targeted to other organelles in which it is degraded at an accelerated rate (Schwartz et al., 1996; Blagoveshchenskaya et al., 2002; Williams et al., 2002). In agreement with other studies performed in T cells (Schwartz et al., 1996), we found that Nef expression accelerated the degradation of mature, endo H-resistant HLA-A2, but did not affect the stability of immature, endo H-sensitive molecules (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200407031/DC1). In addition, we found that two inhibitors of lysosomal protein degradation, ammonium chloride and bafilomycin A1, blocked HLA-A2 degradation. Whereas, lactacystin, a proteasome inhibitor, had no effect on HLA-A2 degradation (Fig. 1 C). Thus, after reaching the TGN, HLA-A2 appears to be targeted for lysosomal degradation in Nef-expressing T cells.

To provide supporting evidence that MHC-I is ultimately targeted to lysosomes, we examined the colocalization of HLA-A2 with LAMP-1, a marker of lysosomal compartments (Fig. 1 D). In T cells expressing Nef, we observed a very small amount of colocalization of HLA-A2 with LAMP-1 (Fig. 1 D, 10). The majority colocalized with markers of the TGN (not depicted). However, when lysosomal degradation was inhib-
ited by treatment of cells with bafilomycin A1, the amount of HLA-A2 colocalizing with LAMP-1 dramatically increased in Nef-expressing cells (Fig. 1 D, compare 10 with 12). These data support the model that MHC-I is ultimately directed to lysosomal compartments in Nef-expressing T cells, where it is rapidly degraded.

**Nef targets HLA-A2 to lysosomes using an AP-1A-dependent pathway**

Transport of lysosomal hydrolases and LAMP-1 to the lysosomes is known to require the APs AP-1 and AP-3, respectively. Interestingly, both of these adaptors are known to interact with HIV-1 Nef. Therefore, to explore a possible requirement for these complexes, we transfected cells with siRNAs directed at the γH9262 subunit of AP-1A and AP-3. As shown in Fig. 2 (A and B), we were able to reduce expression of these molecules in both astrocytic and T cells. In both cell types, we found that inhibiting the expression of AP-1A, but not that of AP-3, reduced the effect of Nef on MHC-I cell surface expression (Fig. 2, C and D). In addition,
AP-1A expression was required for HLA-A2 degradation (Fig. 2 E). However, reducing the expression of μ1A and μ3 did not affect Nef's ability to downmodulate CD4 (Fig. 2, C and D), nor did it affect Nef expression (Fig. 2, A, B, and E). These results suggest that AP-1A expression is specifically required for Nef to disrupt MHC-I cell surface expression and to target it for degradation.

**Nef promotes the formation of a complex containing MHC-I and AP-1**

To determine whether Nef functions as an AP linking MHC-I to AP-1, we immunoprecipitated endogenous HLA-A2 from HIV-infected primary T cells and then immunoblotted for Nef and for endogenous subunits of AP-1. As expected, we detected Nef protein coprecipitating with HLA-A2 (Fig. 3 A). In addition, we also detected the μ and γ subunits of AP-1 coprecipitating with HLA-A2. This result is particularly striking because only ∼25% of the primary T cells were infected in this experiment. The interaction was highly specific based on the fact that the related AP, AP-3, did not coprecipitate with HLA-A2 (Fig. 3 B). Thus, these studies strongly indicate that Nef promotes the formation of a complex containing MHC-I and AP-1.
AP-1 binds to the Nef-MHC-I complex in a dileucine-independent manner

Previous studies that have examined the direct interaction of Nef with AP-1 have found that it depends on the Nef dileucine motif (Bresnahan et al., 1998; Craig et al., 2000; Janvier et al., 2003a,b). However, we found that the dileucine motif was not needed for coprecipitation of AP-1 with MHC-I in CEM T cells treated with adeno-Nef (Fig. 4 A) or HIV (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200407031/DC1). These data are consistent with the fact that the dileucine motif is not necessary for disruption of MHC-I trafficking (Greenberg et al., 1998a; Mangasarian et al., 1999), and suggests that there is an alternative AP-1 binding site in the Nef–MHC-I complex.

To rule out the possibility that the interaction we observed was nonspecific, and to verify that it was dependent on Nef do-
mains important for MHC-I trafficking to lysosomes, we asked whether AP-1 would coprecipitate with MHC-I in cells expressing a Nef mutant (V10E/H900417-26) that was unable to affect MHC-I trafficking and to bind MHC-I (Mangasarian et al., 1999; Williams et al., 2004). As shown in Fig. 4 A, we found that AP-1 did not coprecipitate with HLA-A2 under these conditions.

In addition, we asked whether AP-1 would coprecipitate with an HLA-A2 mutant lacking a tyrosine residue at position 320 that is necessary for responsiveness to Nef (Greenberg et al., 1998b; Le Gall et al., 1998). As shown in Fig. 4 B, this mutant was expressed well (Fig. 4 B, input controls, bottom). However, it was resistant to Nef-dependent MHC-I degradation, coprecipitated Nef less efficiently, and was defective in AP-1 recruitment (Fig. 4 B). Thus, these studies indicate that coprecipitation of AP-1 with HLA-A2 in Nef-expressing cells was highly specific and depended on amino acid sequences in Nef and MHC-I that are functionally important for Nef’s effects on MHC-I cell surface expression.

Recruitment of AP-1 requires both the cytoplasmic tail of HLA-A2 and the NH2-terminal α-helix in Nef

The domains of HIV Nef specifically involved in MHC-I trafficking have been well characterized. It is known that disruption of Nef’s NH2-terminal α-helix by deletion (V10E/A17-26) or point mutation (M37A), mutation of an acidic cluster (E62,Q), or mutation of a polyproline repeat (P72,75A) specifically affects this activity (Greenberg et al., 1998b; Mangasarian et al., 1999). We have recently reported that each of these mutants fails to coprecipitate with MHC-I (Williams et al., 2004) and thus binding to MHC-I may be the primary role of these domains. However, it is also possible that one or more of these domains plays an additional role. To determine whether any of these domains might also be involved in AP-1 recruitment directly, we bypassed the requirement for Nef binding by directly fusing Nef to the COOH terminus of the HLA-A2 cytoplasmic tail (A2/Nef; Fig. 5 A). As expected, Nef was able to reduce the cell surface expression of HLA-A2 in cis (Fig. 5 B, left), and to promote its colocalization with markers of the Golgi apparatus (Fig. 5 C). In addition, the A2/Nef fusion protein was able to efficiently coprecipitate AP-1 from T cell lysates in a manner that was independent of the Nef dileucine motif (Fig. 6 A). However, the fusion protein was not capable of reducing MHC-I surface expression in trans (Fig. 5 B, right). Thus, A2/Nef was a useful reagent to explore which amino acids in Nef and MHC-I were important for AP-1 recruitment to the Nef–MHC-I complex.

As shown in Fig. 6 A, these studies revealed that mutation of amino acids in the NH2-terminal α-helix (A2/V10E/A17-26 and A2/M37A) inhibited coprecipitation of AP-1 with the fusion protein. In contrast, disruption of the acidic (A2/E62,Q) and polyproline (A2/P72,75A and A2/P75,78A) domains in Nef had no effect (Fig. 6, A and B). Surprisingly, deletion of the HLA-A2 cytoplasmic tail (A2ΔTail/Nef) or mutation of tyrosine 320 in the HLA-A2 cytoplasmic tail (A2ΔT320A/Nef) also
dramatically inhibited coprecipitation of AP-1 by the fusion protein (Fig. 6 C). Thus, sequences in the Nef NH₂-terminal/H₉₂₅₁ helix as well as in the HLA-A₂ cytoplasmic tail are necessary to create a functional AP-1 binding site.

Discussion

In sum, we have found that in T cells, Nef disrupts MHC-I trafficking after it reaches the TGN to prevent MHC-I cell surface expression and to promote its degradation in lysosomes. RNAi treatment of Nef-expressing T cells and astrocytic cells revealed that this activity of Nef required the expression of AP-1A, but not AP-3. The requirement for AP-1A was further supported by the fact that we were able to isolate complexes containing MHC-I and AP-1 from Nef-expressing, HIV-infected primary T cells. The dileucine motif in Nef, which is necessary for Nef to directly bind AP-1 in other assay systems, was not needed for AP-1 to bind the Nef–MHC-I complex, suggesting an alternative means of association. Interestingly, when Nef was fused to HLA-A₂, AP-1 was only recruited when the NH₂-terminal α helical domain of Nef was intact and when a full-length, wild-type HLA-A₂ cytoplasmic tail was present. Thus, amino acids within both Nef and MHC-I were necessary to create a binding site for AP-1 in the Nef–MHC-I complex.

The normal function of AP-1 is to sort proteins, such as lysosomal enzyme receptors at the TGN by linking clathrin to the cytoplasmic tails of cargo (for review see Dell’Angelica and Payne, 2001; Robinson and Bonifacino, 2001; Traub, 2003; Robinson, 2004). For example, the mannose 6-phosphate receptor (MPR), which binds soluble lysosomal enzymes, is sorted at the TGN. It is then transported in vesicles to endosomes where the enzymes dissociate and are carried to the lysosomes. The MPRs are then recycled back to the TGN to transport more cargo. Thus, Nef-induced AP-1 binding of MHC-I would be expected to promote TGN accumulation and/or targeting of MHC-I into the endolysosomal pathway (Fig. 7).

Using the yeast two hybrid system (Le Gall et al., 1998; Piguet et al., 1998; Craig et al., 2000; Erdtmann et al., 2000; Janvier et al., 2003b), GST-Nef pull-downs (Bresnahan et al., 1998; Le Gall et al., 1998; Piguet et al., 1998; Janvier et al., 2003a) or overexpression of a Nef-CD8 chimera in 293 cells (Bresnahan et al., 1998), a number of investigators have found that the HIV-1 Nef protein interacts with adaptin subunits and whole adaptin complexes. A consensus binding domain for adaptin protein binding (D/EXXXLL) (Bonifacino and Traub, 2003) can be found in Nef and this motif is required for GST-Nef to pull down AP-1 complexes from mammalian cell lysates. A number of investigators have found that the HIV-1 Nef protein interacts with adaptin subunits and whole adaptin complexes. A consensus binding domain for adaptin protein binding (D/EXXXLL) (Bonifacino and Traub, 2003) can be found in Nef and this motif is required for GST-Nef to pull down AP-1 complexes from mammalian cell lysates. A number of investigators have found that the HIV-1 Nef protein interacts with adaptin subunits and whole adaptin complexes. A consensus binding domain for adaptin protein binding (D/EXXXLL) (Bonifacino and Traub, 2003) can be found in Nef and this motif is required for GST-Nef to pull down AP-1 complexes from mammalian cell lysates.

Figure 6. The cytoplasmic domain of HLA-A₂ and the NH₂-terminal α helix of Nef are both required for AP-1 binding. (A and B) The NH₂-terminal α-helix in Nef is necessary for AP-1 recruitment. CEM-SS cells were transduced with the murine retrovirus encoding HLA-A₂ or the indicated A₂/Nef fusion constructs. Coprecipitating proteins were detected by Western blotting of anti-HLA-A₂ immunoprecipitations. (C) AP-1 binding to the A₂/Nef fusion protein requires tyrosine 3₂₀ (Y₃₂₀) in the cytoplasmic tail of HLA-A₂. The indicated mutations in the A₂/Nef fusion protein were tested for involvement in AP-1 binding using the assay described above. A₂ΔTail is an A₂/Nef fusion protein that lacks the entire HLA-A₂ cytoplasmic tail. All results are representative of at least three independent experiments. White lines indicate that intervening lanes have been spliced out.

Figure 7. Model for MHC-I trafficking in Nef-expressing T cells. To disrupt MHC-I trafficking, Nef first binds to the MHC-I cytoplasmic tail in the secretory pathway. The formation of the Nef-MHC-I complex creates a binding site for AP-1 that is independent of the Nef dileucine motif. This tertiary complex leads to the recruitment of MHC-I into AP-1-positive clathrin-coated vesicles destined for degradation in the lysosomes.
The MHC-I cytoplasmic tail itself does not contain a consensus AP-1 binding site (D/EXXXLL or YXXΦ) (Bonifacio and Traub, 2003). However, the tyrosine residue at position 320 (Y320) that we show is necessary for Nef to recruit AP-1, is also required for the normal targeting of MHC-I to endolysosomal compartments in dendritic cells (Lizee et al., 2003). The mechanism by which MHC-I is normally targeted to these compartments is unknown, and it is interesting to speculate that Nef may be taking advantage of an existing pathway for MHC-I trafficking that is normally only active in certain cell types.

Previous studies describing the mechanism of Nef’s effects on MHC-I have focused on the ability of Nef to accelerate MHC-I endocytosis, rather than on the TGN to lysosomal pathway we describe (Greenberg et al., 1998b; Le Gall et al., 1998, 2000; Piguet et al., 2000; Blagoveshchenskaya et al., 2002; Kasper and Collins, 2003). This is most likely due to the fact that Nef is more active at disrupting transport from the TGN in T cells than in the more commonly used cell lines, such as HeLa cells (Kasper and Collins, 2003). The effect of Nef on endocytosis has been attributed to the small GTPase ADP-ribosylation factor-6 (Blagoveshchenskaya et al., 2002), which is normally involved in MHC-I turnover from the cell surface (Caplan et al., 2002). However, there is new evidence that the effect of ADP-ribosylation factor-6 may be indirect (Larsen et al., 2004).

In sum, our results indicate that in T cells, the primary effect of Nef on MHC-I is to promote TGN retention and to ultimately direct MHC-I to lysosomes. We have provided evidence that Nef accomplishes this by acting as an AP, which stabilizes a physical interaction between AP-1 and MHC-I in order to target MHC-I into the endolysosomal pathway. These results have important implications for understanding HIV disease pathogenesis and for possible pharmaceutical approaches in combating the development of AIDS.

Materials and methods

Cell culture

CEM T cell lines were maintained in R10 (RPMI supplemented with 10% FBS, 10 mM Hepes, 2 mM penicillin, streptomycin, and glucose (P/S/G)). The astrocytic cell lines stably expressing CD4 were maintained in DMEM supplemented with 10% FBS and P/S/G. Stable astrocytic and CEM T cell lines were generated by transduction with MSCV retroviral constructs pseudotyped with VSV-G as described previously (Kasper and Collins, 2003). Primary T lymphocytes were isolated from buffy coats as described previously (Collins et al., 1998).

DNA constructs

A nine-amino acid HA epitope tag (YPYDVPDYA) was added to the NH2 terminus of HLA-A2 and HLA-B71 [just after the leader sequence cleavage site and before the Nael site (Swann et al., 2001)] using the following primers and HLA-B3501 as the DNA template: forward primer, 5′-GGG-AATTCTCTAGAGATCTCCATGACCCCGCAG-3′; reverse primer, 5′-TCC-CGCCGGCATAGCTCGGTACGTCATACGGATAGGACCCGGCCCAG-GTCTCGGT-3′. The PCR product was then digested with EcoRI and Nael and subcloned into a shuttle vector (New England Biolabs, Inc.). Next, the following primers were used to amplify the 3′ region of HLA-A2 and HLA-B71: HLA-A2 forward primer, 5′-GAATATCCATGATCCGGTGACC-3′; HLA-B71 forward primer, 5′-GGGTCCTGAGAATCTCTGAGGAGAACA-CATCG-3′; HLA-A2 reverse primer, 5′-CCGCTCTAGATGCTACCTCAAATGTTACG-3′; HLA-B71 reverse primer, 5′-CTGGGCCGCGCCATACCCATCTGCTGTCCT-3′. The resulting PCR products were digested with Nael and cloned into the same sites in MSCV.A2/H8A2. MSCV.HLA-A2 Y320A was cloned by amplifying a portion of HLA-A2 Y320A (Swann et al., 2001) using the following primers: forward primer, 5′-GCAAGCTCAGACCAACCCAG-3′; reverse primer, 5′-CCGCTCGAAGACCAACCCACG-3′. The PCR product was then digested with PmlI and XhoI and cloned into the same sites in MSCV.A2/H8A2 to replace the wild-type sequence. An adenoviral vector expressing HXB Nef Ll14_164_166AA was generated by PCR mutagenesis of the HIV molecular clone, HXB-P1 (Chen et al., 1996), using the following primers: mutant forward primer, 5′-AATTCCTCAGAATCTCCTCAGACGCCGAG-3′; reverse primer, 5′-GCTCTAGATGCTAGAGATTTTC-3′. The PCR product was then digested with EcoRI and Nael and cloned into the same sites in MSCV.A2/H8A2 to replace the wild-type sequence.

An adenoviral vector expressing HXB Nef Ll14_164_166AA was generated by PCR mutagenesis of the HIV molecular clone, HXB-P1 (Chen et al., 1996), using the following primers: mutant forward primer, 5′-GAAGGAGAGAAGCAACCCGGCGGCACCCCTGTGGAGGATAGG-3′; with reverse primer (3′-hxb-xba), 5′-GCTCTAGATGCTAGAGATTTTC-3′; and mutant reverse primer, 5′-TCCATGACCCGTCGACGAGGATCTGGTTTGTGCTCTGTCTTTT-3′; with forward primer (5′-nef), 5′-CGGATCCATGATGCTAGAGATTTTC-3′; with reverse primer (3′-hxb-xba), 5′-GCTCTAGATGCTAGAGATTTTC-3′; with reverse primer (3′-hxb-xba), 5′-GCTCTAGATGCTAGAGATTTTC-3′; and mutant reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; with forward primer (5′-nef), 5′-CGGATCCATGATGCTAGAGATTTTC-3′; and mutant reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; with forward primer (5′-nef), 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; and mutant reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′. The resulting mutant PCR products were mixed and reamplified with 3′-hxb-xba and 5′-nef primers, digested with XbaI and XhoI and cloned into the same sites of HXB-P1. The mutated Nef was then amplified and cloned into an adenoviral vector shuttle plasmid as described previously (Swann et al., 2001). The adenoviral vector expressing Nef V10E_E137_26 has been described previously (Williams et al., 2004).

An HIV molecular clone containing the Nef Ll14_164_166AA mutation was generated by PCR mutagenesis of the HIV molecular clone, HXB-EP (Chen et al., 1996), using the following primers: mutagen forward primer, 5′-GATC-AAAGGGAGAGAAGCAACCCGGCGGCACCCCTGTGGAGGATAGG-3′; and reverse primer, 5′-GCTCTAGATGCTAGAGATTTTC-3′; and with reverse primer (3′-hxb-xba), 5′-GCTCTAGATGCTAGAGATTTTC-3′; and reverse primer, 5′-GCTCTAGATGCTAGAGATTTTC-3′; and with reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; with forward primer (5′-nef), 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; and mutant reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′; and mutant reverse primer, 5′-ATGCAGGCTCACAGGGTGTTTGCACCTTTACGGC-3′.
IRES GFP construct was generated using the following primers 5′ (pMIG) (Van Parijs et al., 1999) in a three-way ligation. The control HLA-A2 was digested with BamHI and SpeI, the Nef PCR product was digested with GCGAATTCTCAGCAGTTCTTGAAGTACTC-3′.

Mix-L [35S] (Adeno-transduced CEM T cells were collected and washed twice with D-PBS dia was added. Cells were harvested at 24–72 h after transduction.

From the A2/Nef template using the following mutant primers: 5′ Nef 3′ (GATCCACCATGGTACCGTGCACG-3′).

The resulting construct was then digested with XhoI and BglII and ligated into the BglII and HpaI sites of pMIG. A2 Y320A/Nef was generated with the primers; 5′-AGAAAAGGAGGGAGCGCCTCTCAGGCTGCA-3′, 5′-GGTCACTAGTCACTTCAACAAGCTGGGAGAAGAAGA-3′. This PCR product was digested with BamHI and ligated into the BglII and Hpal sites of pmig. A2 Y320A/Nef was generated from the A2/Nef template using the following mutant primers: 5′-AGAAGAGGAGGGAGCGCCTCTCAGGCTGCA-3′, 5′-GGTCACTAGTCACTTCAACAAGCTGGGAGAAGAAGA-3′.

MSCV-EYFP-Golgi was constructed as follows: EYFP-Golgi (CLONTECH Laboratories, Inc.) was digested with NheI and HpaI. This fragment was inserted into the XhoI and SpeI sites of the bicistronic retroviral vector expressing the IRES GFP cassette (pMIG) (Van Parijs et al., 1999) in a three-way ligation.

Viral transduction of T cells
CD8-depleted PHA-activated primary T cells or CEM-SS cells were transduced with VSV-G-pseudotyped HIV molecular clones (HX8-EP nef and nef1), VSVG-pseudotyped murine retroviruses or adenoviral vectors as described previously (Collins et al., 1998; Swann et al., 2001; Kasper and Collins, 2003). Recombinant replication defective E3-deleted adenovirus expressing HIV-1 NL4-3 Nef, Nef V10E, Δ17-26, Nef L164,165AA, or no insert was generated by the University of Michigan Vector Core. For adenoviral transduction, CEM cell lines were resuspended in RPMI, 2% FBS, P/S/G and 10% dialyzed FBS (Invitrogen) for 15 min at 37°C. Cells were collected and washed once with D-PBS and lysed in 1 ml of lysis buffer [50 mM Tris, pH 8.1, 1% NP-40 (vol/vol), 5 mM MgCl₂, 1 mM PMSF] for 30 min on ice, followed by centrifugation to remove insoluble material. Cellular lysates were precleared overnight at 4°C with 1.5 μg of mouse IgG1 (BD Biosciences) and 30 μl of protein A/G agarose (50% slurry; Calbiochem). For immunoprecipitations, 5 μg of anti-HLA-A2 (B87.2) or 3.5 μg of anti-transferrin receptor antibody (α-Transferrin; Oncogene) was added to the precleared lysate, followed by the addition of 30 μl of protein A/G agarose beads. After 2 h at 4°C, the beads were washed three times with 1 ml RIPA buffer (Kasper and Collins, 2003). Endo H [New England Biolabs, (NEB)] or neuraminidase [New England Biolabs, (NEB)] digestion of immunoprecipitated material was performed according to the manufacturer’s protocols. Samples were separated by 10% SDS-PAGE and the gels were dried and exposed to BioMaxMS films at −80°C. Radio-labeled proteins were quantitated using a Phosphor storage screen and Typhoon Scanner followed by processing with ImageQuaNT software.

Immunofluorescence microscopy
CEM cells were allowed to adhere to chambered glass slides coated with poly-L-lysine (Sigma-Aldrich) for 1 h at 37°C. Cells were then fixed at RT for 15 min in D-PBS + 2% PFA. Next, cell membranes were permeabilized by incubation in D-PBS + 0.2% Tween 20 for 15 min at 37°C. Cells were washed twice with wash buffer (D-PBS, 10 mM Hepes, 10% goat serum [Sigma-Aldrich], 0.025% sodium azide) followed by blocking in wash buffer (adjusted to 10% goat serum and 10% human serum; Sigma-Aldrich) for 30 min on ice. For Fig. 1, mouse mAbs were used to detect LAMP-1 (clone HA3; BD Biosciences; 1:1,000) and HLA-A2 (B87.2, 20 μg/ml). Isotype-specific secondary antibodies were used to distinguish between these antibodies (anti-mouse IgG1 Alexa-Fluor488 [Becton Dickinson; Biocytin Probes] and anti–mouse IgG2b Alexa-Fluor488 [1:250; Molecular Probes], respectively). All antibody incubations were performed in wash buffer on ice for 20 min. Slides were mounted with coverslips using ProLong antifade reagent (Molecular Probes). Images were collected using a confocal microscope (model LSM 510; Carl Zeiss Microlmaging, Inc.) and processed using Adobe Photoshop 6.0. The University of Michigan Vector Core’s adenovirus-hA-HLA-A2 cells were resuspended in fresh R10 containing 100 μM bafloxicin A1 (Sigma-Aldrich) or solvent alone (DMSO) and incubated at 37°C for 3 h before adherence.

RNAi treatment
The following duplex siRNAs (Ambion) were used in this study: siGFP, sense 5′-GCGCUGCCCGAAGUUCAUCACT-3′; antisense 5′-GAUAGCC UUUCGUGCUAAGCT-3′; siβ3, sense 5′-GGACUACUUUGGUAGG UGTT-3′, antisense 5′-ACACUCAAAAAGUGUACCTTG-3′. The μA siRNA was described previously (Hirst et al., 2003). Astrocytoma cells stably expressing CD3 (CD3-CD4) were transfected with annealed duplex siRNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. In brief, cells were plated onto 6-well plates (10% of well full) and the next day each well was transfected with 0.16 nmol siRNA and 4 μl of Lipofectamine 2000 reagent. 24 h later, cells were replated onto 6-well plates (10% cells per well) and the cells were transfected with siRNA again 24 h after replating. 4–6 h after transfection, the media was removed and the cells were transfected with adenovirus (MOI = 50). Cells were harvested and analyzed 24 h after transduction.

For experiments using CEM T cells, the cells were first transfected with adenovirus (MOI = 50). 24 h later, 5 × 10⁴ cells were electroporated with 1 nmole siRNA according to the manufacturer’s protocol (Amazk Biosystems). The cells were then incubated for 48 h before FACS and Western blot assays. For Fig. 2 E, the cells were subjected to an initial siRNA transfection 1 d before the adenoviral transduction, and the experiment was then performed as described above.

Antibodies and Western blot analyses
IP-Western experiments were performed as described previously (Williams et al., 2004). H9A-HLA-A2 was detected using the anti-HA antibody, HA.11 (1:5,000; Covance). Co precipitating Nef protein was detected by Western blotting using antibodies obtained via the NIH AIDS Research and Reference Receptor Repository, Division of AIDS, NIAID, NIH: polyclonal anti-Nef antibody [2949, 1:5,000], a gift from R. Swanstrom, The University of North Carolina at Chapel Hill, Chapel Hill, NC; Shugars et al., 1993) or monoclonal anti-Nef (AG11, 1:500) for IP-Westerns and 1:10,000 for crude lysate, a gift from J. Hoxie, University of Pennsylvania, Philadelphia, PA; Chang et al., 1998). The adaptin subunit, α- adaptin (1:100 for IP-Westerns; 1:1,000 for crude lysate) was detected using RY/1 (1:2,500, a gift from L. Traub, University of Pittsburgh, Pittsburgh, PA; Traub et al., 1995). Other antibodies to AP subunits were purchased from BD Biosciences: γ-adaptin (1:100 for IP-Westerns; 1:1,000 for crude lysate); β-adaptin (1:100 for IP-Westerns; 1:1,000 for crude lysate); and μ3 (p47A, 1:500). The HLA-A2-specific mAb, B87.2 (Parham and Brodsky, 1981), and anti-Nef antibody, AG11 (Chang et al., 1998), were purified from ascites fluid as described previously (Kasper and Collins, 2003).

Online supplemental material
Fig. S1 shows that the addition of an HA tag does not affect MHC-I folding, maturation through the secretory pathway and refolding. Online supplemental material demonstrating that the dileucine motif is not required for the Nef-MHC-I interaction is available at http://www.jcb.org/cgi/content/full/jcb.200407031/DC1.

We are grateful to Dr. Linton Traub for antibody to μ1 to the NIH AIDS Research Repository for antibodies, to the University of Michigan vector core for adenovirus preparation, to Dr. Randy Schekman for helpful suggestions and to Drs. John Moran and Mark Benson for critical reading of the manuscript.

This work was supported by National Institutes of Health grant ROI AI46998. M.R. Kasper was supported by the University of Michigan Genetics Training Program. M. Williams and J. Roeth were supported by the Univer...
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