The Tyrosine Binding Pocket in the Adaptor Protein 1 (AP-1) μ1 Subunit Is Necessary for Nef to Recruit AP-1 to the Major Histocompatibility Complex Class I Cytoplasmic Tail

To evade the anti-human immunodeficiency virus (HIV) immune response, the HIV Nef protein disrupts major histocompatibility complex class I (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 (YXXφ motifs) via physically separate binding sites. In the case of the Nef-MHC-I complex, a tyrosine in the human leukocyte antigen (HLA)-A2 cytoplasmic tail (320YSQA) and a methionine in Nef (Met20) 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 Ala324 and Asp327 in the cytoplasmic tails of HLA-A and (but not HLA-C and HLA-E) molecules also stabilized AP-1 binding. Finally, mutation of the tyrosine binding pocket in the μ1 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.

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 result from variations in MHC-I cytoplasmic tail sequences. There are three key amino acids in the cytoplasmic tails of Nef-responsive MHC-I molecules (Tyr320, Ala324, and Asp327). 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, E62Q/E63Q/E64Q/E65Q, P72A/P75A, and P75A/P78A) 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 (β1, γ, σ1, μ1A, and/or μ1B) (9–11) and is thought to promote trafficking from the trans–Golgi network to endosomes (12, 13). There are physically separate binding sites for cytoplasmic sorting signals on AP-1; μ1 binds YXXφ signals (Y, tyrosine; φ, bulky hydrophobic amino acid) (14, 15), the β1 subunit (16), and a hemi-complex composed of the σ and γ subunits (17) bind dileucine motifs.

AP-1 (and Nef) co-precipitates with MHC-I in HIV-infected primary T cells (8). In addition, siRNA to AP-1 dramatically inhibits MHC-I down-modulation caused by Nef (8, 18). Nef

* The abbreviations used are: CTL, cytotoxic T-lymphocytes; HLA, human leukocyte antigen; MHC-I, major histocompatibility complex class I proteins; IRES, internal ribosomal entry site; PLAP, placental-like alkaline phosphatase; AP-1, adaptor protein 1; siRNA, small interfering RNA; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; TBPM, tyrosine binding pocket mutant; YFP, yellow fluorescent protein; MSCV, murine stem cell virus.

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binds AP-1 in yeast two-hybrid and glutathione S-transferase pulldown 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 Tyr320 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 Tyr320 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 Tyr320, Ala324, and Asp327 in the MHC-I cytoplasmic tail. Creation of a sequence that resembled a canonical AP-1 signal (substituting YSQA for YSQV 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 down-modulation and for AP-1 to bind HLA-A2. In sum, these data support the model that multiple Nef domains work together to allow Tyr320 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 Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM penicillin, streptomycin, and glutamine. CEM T4-lym- phoblastoid cell lines were cultured in RPMI 1640 that was supplemented with 10% fetal bovine serum, 10 mM HEPES, and 2 mM penicillin, streptomycin, and glutamine. Selection of transduced stable CEM cell lines was performed using 2.5 μg/ml puromycin (Fisher) or 1 mg/ml Geneticin (Invitrogen).

Preparation of Expression Plasmids Containing A2/Nef mutant primers previously described (8). 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 polyethyleneimine, M, 25,000 (Polysciences Inc.).

Viral Transduction of CEM Cell Lines—Stable CEM cell lines were transduced with control or Nef-expressing adenovirus as described previously (7). Multiplicity of infection for FACS analysis was 100–500 (based on 293-cell infectivity, which is greater than CEM infectivity). Multiplicity of infection for immunoprecipitation and Western blotting was 50–100. Transductions in low serum media ranged from 4 to 7 h. Retroviral supernatants were prepared as previously described (19, 21) using a bicistronic retroviral vector expressing an IRES GFP cassette (pMIG) (21) except that they were pseudotyped with pCMV VSV-G (Dr. Nancy Hopkins, MIT). 1 × 10⁶ CEM T cells were spin-transduced with the retroviral supernatants by centrifuging at 2500 rpm at room temperature in a tabletop centrifuge for 2 h with 8 μg/ml Polybrene.

Flow Cytometry and Antibodies—72 h post-transductions, cells were stained in FACS buffer (phosphate-buffered saline, 2% human serum, 1% HEPES, 1% NaCl) with either an anti-HLA-A2 antibody (BB7.2 (22) 1:100) or anti-CD4 antibody.
(Serotec; 1:100) and, when appropriate, an anti-human PLAP antibody (DAKO; 1:500). The fluorescently conjugated secondary antibody, goat anti-mouse-phycocerythrin (Invitrogen, 1:250), was used for Figs. 1, 3, 5, 6, and 7. Isotype-specific fluorescently conjugated secondary antibodies were utilized in Fig. 3. The secondary antibody for BB7.2 was goat anti-mouse IgG2a-specific-PE-mM MgCl2. Phenylmethylsulfonyl fluoride and protease inhibitors from Amersham Biosciences.

The membranes were washed in 0.5 mg/ml biotin (EZ-Link sulfo-NHS-LC-Biotin, Pierce) for 1 h. 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, whereas the remaining two-thirds were re-purified with avidin agarose (Calbiochem).

Immunoprecipitation and Western blot analysis was performed similarly to previous publications (7, 8) with the following exceptions; a 16-h 20 mM ammonium chloride treatment was performed, the cross-linking step with dimethyl 3,3’-dithiobispropionimidate-2HCl was omitted, and cells were lysed with digitonin. The digitonin lysis buffer was 1% digitonin (Wako), 100 mM NaCl, 50 mM Tris, pH 7.0, 1 mM CaCl2, and 1 mM MgCl2. Phenylmethylsulfonyl fluoride and protease inhibitor tablets were included as before (8). Lysates were normalized for total protein and GFP transduction rates, when appropriate, before 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 μ1 (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; immunoprecipitate, 1:500; whole cell lysate, 1:1000), and anti-HA (Covance; 1:1000). The secondary antibody for AG11 and anti-HA was goat anti-mouse IgG1, horseradish peroxidase (HRP; Zymed Laboratories Inc.; 1:25,000–50,000), for RY/1 was rabbit anti-mouse IgG-HRP (Zymed Laboratories Inc.; 1:25,000–50,000), and for anti-AP-1 adaptin subunit γ was goat anti-mouse IgG-HRP (1:25,000–50,000). The membranes were developed with the ECL Plus Western blotting detection kit from Amersham Biosciences.

RNA-mediated Interference Treatment—Duplex siRNAs (Ambion) were transfected into astrocytoma cells (373MG) as described previously (8). Briefly, on day 1, 1.25 × 10^6 373MG cells were plated onto a 100-mm dish. The next day the cells were transfected using 0.64 nmol of duplex siRNA and 16 μl of Lipofectamine 2000. On day 3 the cells were re-plated onto 6-well plates. On day 4 the cells were re-transfected with siRNA using 0.16 nmol of duplex siRNA and 4 μl of Lipofectamine 2000. Fours hours after transfection, retroviral supernatants and 8 μg/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 μ1A siRNA used has been described previously (26).

RESULTS

Mutating Three Amino Acids in the Cytoplasmic Tail of HLA-A2 Abrogated Nef-induced Down-modulation 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 among 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 Fig. 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). In addition, Tyr^{20} was mutated to cysteine to mimic the HLA-C molecule, and Ala^{324} 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. 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 Fig. 1, B 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 down-modulation was observed when amino acids Gln^{322}, Ser^{325}, and Ser^{326} were mutated (Figs. 1, B and C). Interestingly as seen in Fig. 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 HLA-A2 in Nef-expressing cells (p value = 0.01). This substitution creates a sequence more similar to a canonical AP-1 binding motif (YXX{b}) 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 HLA-A2 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 Fig. 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 down-modulated by Nef, did not recruit the μ1 or γ subunits of AP-1 (Fig. 2A, lanes 3, 12, and 15). Interestingly, Nef was still able to bind Y320C, A324E, and D327A in the absence of AP-1.
FIGURE 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 under “Materials and Methods.” The cells were stained 72 h 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 ± S.D. 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, S.D., and paired t tests were calculated from three independent experiments.
Nef Uses the AP-1 Tyr Binding Pocket to Down-modulate MHC-I

FIGURE 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 (IP) with the anti-HLA-A2 antibody BB7.2 as described under "Materials and Methods." Proteins that stably interacted with HLA-A2 were detected by Western blotting (WB) as indicated. Relative protein levels before 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 are shown in A.

(Fig. 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 (Fig. 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 (Fig. 2A, lanes 6, 7, 13, and 14).

Perhaps because of the increased hydrophobicity of valine compared with 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 (Fig. 2B, compare 4d with 8d), but Nef dramatically enhanced this interaction (Fig. 2B, compare 8d and 9d). The combination of the need for Tyr320 and the enhanced interaction with A323V suggested that Nef recruited AP-1 by utilizing the canonical YXX\(\phi\) signal binding pocket found in the AP-1 \(\mu 1\) subunit.

The \(\mu 1\) Subunit of AP-1 Uses Its Canonical Tyrosine Binding Pocket for Nef-induced Down-modulation of HLA-A2—To determine whether Nef recruited AP-1 to bind the YSQA sequence in HLA-A2 via its YXX\(\phi\) 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 \(\mu 1\) 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 IRES. We then used flow cytometry to demonstrate that the \(\mu 1\) TBPM indeed acted as a dominant negative inhibitor of Nef (Fig. 3A, compare PLAP- to PLAP\(^+\) in the lower two FACS plots). This effect, which was quantified in Fig. 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 immunoprecipitation-western blot experiment shown in Fig. 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 (Fig. 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 Amino-terminal \(\alpha\)-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 \(\alpha\)-helical domain from amino acids 17 through 26 in Nef in the lower two FACS plots). This effect, which was quantified in Fig. 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 immunoprecipitation-western blot experiment shown in Fig. 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 (Fig. 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 Amino-terminal \(\alpha\)-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 \(\alpha\)-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 (Fig. 4A) and transiently expressed each Nef mutant in CEM T cells using a murine retroviral vector that also expressed GFP. As shown in Fig. 4B, we found that only Met20 was needed for HLA-A2 down-modulation. In comparison, none of the \(\alpha\)-helical mutants was 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 (Fig. 4C).

Nef acidic (Glu62–65) and Polyproline Domains (Pro75/78) 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. (8) utilized an A2/Nef fusion protein in which Nef was fused to the end of the cytoplasmic domain to bypass the requirement for Nef binding (Fig. 5A). This analysis revealed that both Met20 in Nef and the tyrosine residue in the MHC-I cytoplasmic tail are necessary for AP-1 recruitment. Three additional regions in Nef are also needed for MHC-I down-modulation: an acidic region (Glu62–65), an aspartate residue (Asp123), and a polyproline repeat (Pro72/75/78). 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 (Fig. 5, B and C). A partial explanation may be that expression of some of these mutants was slightly lower than wild type A2/Nef (Fig. 5D). However, the defect in AP-1 binding shown in Fig. 5E for M20A in particular (compare lanes 2 and 3) was far greater than the differences in protein expression shown in Fig. 5D.

Surprisingly, in contrast to our previously published results, we consistently observed that mutation of the acidic (Glu62–65) and polyproline (Pro75/78) domains also disrupted AP-1 binding (Fig. 5E, lanes 4 and 5), although these domains were not required to the same extent as Met20. 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 cross-linker, 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 cross-linker

The A2/Nef Fusion Protein Contains Two Active Trafficking Signals—As shown in Fig. 5, mutating Met\(^{20}\) in the A2/Nef fusion protein abrogated AP-1 recruitment (Fig. 5E, lane 3), but, unlike trans-Nef, the mutant A2/Nef fusion protein was still down-modulated relative to wild type HLA-A2 (Fig. 5, B 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 the Nef 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 (Fig. 6, A and B, and quantified in B and D, respectively). Additionally, the double mutant, which lacked both the dileucine and Met\(^{20}\) (A2/M20AxLLNef) (or the entire \(\alpha\) helical domain, including Met\(^{20}\) (A2/V10E\(\Delta\)17–26xLL)), was even more defective, retaining only about 10% of wild type activity (Fig. 6, C and D). Thus, in the fusion protein there are two trafficking signals, one that depends on Tyr\(^{320}\) in MHC-I and Met\(^{20}\) 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 (Fig. 6, A and B). The oligomerization domain (Asp\(^{123}\)) was absolutely required whether or not the dileucine motif was present (Figs. 5, B and C, and Fig. 6, A and B).

The pattern of AP-1 recruitment for doubly mutated fusion proteins shown in Fig. 6E perfectly matched the singly mutated fusion protein recruitment pattern seen in Fig. 5E and correlated well with HLA-A2 down-modulation (Fig. 6B). Thus, it was the presence of the dileucine motif in the fusion proteins that masked a requirement for these domains in Fig. 5, B 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 (Fig. 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 \(\mu\)1A subunit (Fig. 7A). As shown in Fig. 7, B 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 Figs. 7, B and C, \( p = 0.008 \)). In contrast, when Tyr\(^{320} \) 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 (Fig. 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 with wild type HLA-A2, A2-YFP was exported to the cell surface at a somewhat slower rate (Fig. 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 (Fig. 7D).

Finally, we also asked whether both motifs affected the rate of internalization of the fusion protein. As shown in Fig. 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 (A2 Y320A/L164A/L165A) 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 \( /H^9262 \) subunit. Interestingly, AP-1 recruitment to the HLA-A2 cytoplasmic tail tyrosine required additional amino acids (\( ^{320}YXXAAXXD^{327} \)) beyond the usual YXX\( \phi \) signal. The Nef protein stabilized this unusual interaction via several domains. A methionine within Nef (Met\(^{20} \)) was absolutely required. Additionally, Nef acidic (Glu\(^{62–65} \)) and polyproline (Pro\(^{75–78} \)) 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 \( \mu1 \) TBPM provided evidence that the presence of wild type AP-1 was able to enhance the interaction among the three proteins. Finally, we demonstrated that fusion
of Nef to the HLA-A2 cytoplasmic tail activated a second trafficking signal composed of the Nef 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 Tyr320, Ala324, and Asp327 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 YXXΦ signal, also bound Nef better and resulted in more Nef-dependent down-modulation. These data confirmed prior reports that mutating this region to YSQ(I/L)323 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-1 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 μ1 subunit, which interacts with YXXΦ 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 μ1 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-AP-1 complex. Interestingly, in the presence of AP-1 μ1 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 Cytosolic 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
Nef Uses the AP-1 Tyr Binding Pocket to Down-modulate MHC-I

A

| siRNA     | HLA-A2 | A2/Nef | A2/\times LL | Y320A/Nef | Y320A/\times LL |
|-----------|--------|--------|--------------|-----------|----------------|
| none      | µ1     | none   | µ1           | none      | µ1             |
| ctrl      | µ1     | ctrl   | µ1           | ctrl      | µ1             |

WB: µ1

B

siRNA: Negative Control AP-1 µ1

A2/Nef

A2/\times LL

Y320A/Nef

Y320A/\times LL

HLA-A2

C

Fold Down-Modulation

![Graph showing fold down-modulation of MHC-I expression](image)

D

% A2 cell surface transport

![Bar graph showing percentage of A2 cell surface transport](image)

E

Percent Remaining on Surface

![Graph showing percentage remaining on surface over time](image)
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-glutathione S-transferase 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 bypassed this step by directly fusing Nef to the cytoplasmic tail domain, revealed that a region of the amino-terminal α-helical domain (and Met20 within this domain) was also required for AP-1 recruitment to the Nef-MHC-I complex (8). Using new assay conditions, which omitted protein cross-linker, we were able to also detect a role for the acidic domain (Glu62–65), and more dramatically, for the polyproline (Pro75/78) 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 cross-linker (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 Tyr320 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 Fig. 8. First, Tyr320 may be buried in the tyrosine binding pocket of AP-1 and may not directly interact with Nef. In this scenario Tyr320 may be needed to maintain the conformation of the MHC-I tail that Nef binds (Fig. 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 (Fig. 8A). Another possible explanation for how Tyr320 might interact with both proteins is that a conformational change occurs that allows sequential interactions to occur (Fig. 8B).

The Role of the Nef 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 the Nef 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 (Fig. 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 sup-

FIGURE 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 (si1). B, siRNA directed against AP-1 affects the down-modulation of A2/Nef fusion protein variants only when Tyr320 is present. 373MG astrocytoma cells were transduced as in Fig. 5B. Shaded curve, A2 only; black line, A2/Nef variant; gray line, A2 negative. C, quantitation of AP-1 decrease in surface expression. The mean ± S.D. is shown (n = 4). Significant differences are denoted with an asterisk. p values for paired t test; A2/Nef, 0.0006; A2/xLL, 0.0098. D, transport assay. CEM SS cells were transduced as described in Fig. 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 in1hi s

FIGURE 8. Proposed models of the Nef-MHC-I-AP-1 complex. A, Tyr320 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 Tyr320 to alanine (A) allosterically disrupts the Nef binding site, and AP-1 is no longer recruited. Cysteine (C) substitutions for Tyr320 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, Tyr320 binds Nef and AP-1 sequentially. In this model Nef binds to MHC-I via Tyr320 early in the secretory pathway (35), and subsequently, upon AP-1 binding in the trans-Golgi network, Tyr320 interacts only with AP-1.
ports the model that trans-Nef has a limited effect on 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 down-modulates 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 among these proteins will facilitate the development of pharmaceuticals, which may someday help combat AIDS.

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