A Basic Patch on α -Adaptin Is Required for Binding of Human Immunodeficiency Virus Type 1 Nef and Cooperative Assembly of a CD4-Nef-AP-2 Complex^{∇†}

Rittik Chaudhuri,^{1,2} Rafael Mattera,¹ O. Wolf Lindwasser,¹
Margaret S. Robinson,² and Juan S. Bonifacio^{1*}

Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892,¹ and Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0XY, United Kingdom²

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A critical function of the human immunodeficiency virus type 1 Nef protein is the downregulation of CD4 from the surfaces of infected cells. Nef is believed to act by linking the cytosolic tail of CD4 to the endocytic machinery, thereby increasing the rate of CD4 internalization. In support of this model, weak binary interactions between CD4, Nef, and the endocytic adaptor complex, AP-2, have been reported. In particular, dileucine and diacidic motifs in the C-terminal flexible loop of Nef have been shown to mediate binding to a combination of the α and $\sigma 2$ subunits of AP-2. Here, we report the identification of a potential binding site for the Nef diacidic motif on α -adaptin. This site comprises two basic residues, lysine-297 and arginine-340, on the α -adaptin trunk domain. The mutation of these residues specifically inhibits the ability of Nef to bind AP-2 and downregulate CD4. We also present evidence that the diacidic motif on Nef and the basic patch on α -adaptin are both required for the cooperative assembly of a CD4-Nef-AP-2 complex. This cooperativity explains how Nef is able to efficiently downregulate CD4 despite weak binary interactions between components of the tripartite complex.

CD4, a type I transmembrane glycoprotein that serves as a coreceptor for major histocompatibility complex class II (MHC-II) molecules, is expressed on the surfaces of helper T lymphocytes and cells of the monocyte/macrophage lineage (8). Primate immunodeficiency viruses gain access to these cells by virtue of the interaction of the viral envelope glycoprotein (Env) with a combination of CD4 and a chemokine receptor (63). This interaction causes a conformational change within the Env protein that promotes the fusion of the viral envelope with the plasma membrane. Upon the delivery of the viral genetic material into the cytoplasm of the host cells, one of the first virally encoded proteins to be expressed is Nef, an accessory factor that modulates specific signal transduction and protein-trafficking pathways in a manner that optimizes the intracellular environment for viral replication (reviewed in references 21, 39, and 65). Perhaps the best characterized function of Nef is the downregulation of CD4 from the surfaces of the host cells (6, 22, 29, 45). CD4 downregulation prevents superinfection (6, 41) and enhances virion release (19, 38, 48, 66, 76), thereby contributing to the establishment of a robust infective state (24, 72).

The mechanism used by the Nef protein of human immunodeficiency virus type 1 (HIV-1) to downregulate CD4 has

been the subject of extensive study, but only recently have the molecular details of this process begun to be unraveled. It is generally acknowledged that HIV-1 Nef accelerates the internalization of CD4 from the plasma membrane by linking the cytosolic tail of the receptor to the clathrin-associated endocytic machinery (1, 12, 20, 34, 40, 64). In support of this model, a hydrophobic pocket comprising W57 and L58 on the folded core domain of Nef binds with millimolar affinity to the cytosolic tail of CD4 (28) (all residues and numbers correspond to the NL4-3 variant of HIV-1 Nef used in this study). In addition, a dileucine motif (ENTSL, residues 160 to 165) (10, 16, 26) and a diacidic motif (D174 and D175) (2) on the C-terminal flexible loop of Nef mediate an interaction of micromolar affinity with the clathrin-associated, heterotetrameric (α - $\beta 2$ - $\mu 2$ - $\sigma 2$) adaptor protein 2 (AP-2) complex (12, 20, 40, 49). These interactions draw CD4 into clathrin-coated pits that eventually bud inwards as clathrin-coated vesicles (11, 27). Internalized CD4 is subsequently delivered to endosomes and then to lysosomes for degradation (3, 23, 59, 64).

Despite progress in the understanding of the mechanism of Nef-induced CD4 downregulation, several important aspects remain to be elucidated. Previous studies have shown that the Nef dileucine and diacidic motifs interact with a combination of the α and $\sigma 2$ subunits of AP-2 (referred to as the α - $\sigma 2$ hemicomplex) (12, 20, 40, 49), but the precise location of the Nef binding sites is unknown. It also remains to be determined whether Nef can actually bind CD4 and AP-2 at the same time. Indeed, the formation of a tripartite CD4-Nef-AP-2 complex in which Nef links the cytosolic tail of CD4 to AP-2 has long been hypothesized but has never been demonstrated experimentally. Given the relatively weak affinity of Nef for the CD4

* Corresponding author. Mailing address: Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Building 18T, Room 101, National Institutes of Health, Bethesda, MD 20892. Phone: (301) 496-6368. Fax: (301) 402-0078. E-mail: juan@helix.nih.gov.

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tail (28) and AP-2 (12, 40), it is unclear how such a complex could assemble and function in CD4 downregulation.

In this study, we have addressed these issues by using a combination of yeast hybrid, *in vitro* binding, and *in vivo* CD4 downregulation assays. We report the identification of a candidate binding site for the Nef diacidic motif on the AP-2 complex. This site, a basic patch comprising K297 and R340 on α -adaptin, is specifically required for Nef binding and Nef-induced CD4 downregulation. We also show that the Nef diacidic motif and the α -adaptin basic patch are required for the cooperative assembly of a tripartite complex composed of the CD4 cytosolic tail, Nef, and the α - σ 2 hemicomplex. The cooperative manner in which this complex is formed explains how Nef is able to efficiently downregulate CD4 from the plasma membrane despite weak binary interactions between the components of this complex.

MATERIALS AND METHODS

Y3H assays. The pBridge.Nef. σ 2, pBridge.tyrosinase. σ 2, and pGADT7. α plasmids have been described previously (12, 33, 40, 49). All mutations of these plasmids were carried out by site-directed mutagenesis, using the QuikChange II kit (Stratagene, La Jolla, CA) and appropriate primers. The open reading frames (ORF) of these and all other constructs used in this study were verified by nucleotide sequence analysis. Yeast three-hybrid (Y3H) assays were performed as described before (12, 33, 40, 49). Briefly, the *Saccharomyces cerevisiae* HF7c strain was cotransformed with pBridge- and pGADT7-based vectors by the lithium acetate procedure, and double transformants were selected on dropout agar plates lacking Leu, Trp, and Met (+His plates). After 4 days, pooled colonies from each transformation were resuspended in water and the suspensions were normalized to equivalent optical densities (ODs; 0.1 OD units at 600 nm) and transferred onto three sets of plates: +His plates; those lacking Leu, Trp, Met, and His (–His plates); and those lacking Leu, Trp, Met, and His and supplemented with 1 mM 3-amino-1,2,4-triazole (3AT; –His + 3AT plates). Wild-type HIV-1 NL4-3 Nef (or the cytosolic tail of mouse tyrosinase) was expressed as a GAL4BD fusion protein from the pBridge vector (Clontech, Mountain View, CA), along with wild-type or mutant rat σ 2-adaptin. Wild-type or mutant rat α -adaptin was expressed as a GAL4AD fusion protein from pGADT7 (Clontech). The amino acid sequences of rat and human σ 2 are 100% identical, while the trunk domains of rat and human α C are 99.1% identical and 99.8% similar. Colony growth on the three sets of plates was analyzed 3 days later. The growth of the yeast on the –His plates was indicative of an interaction between the GAL4BD and GAL4AD fusion proteins. Growth on the –His + 3AT plates indicated stronger interactions. Each Y3H experiment was performed a minimum of three times.

Y4H assays. To generate pBridge.Nef, an EcoRI-Sall fragment containing the Nef ORF was subcloned from pNL4-3 Nef.IRES.GFP (12, 40) into the corresponding sites of multiple cloning site 1 (MCS1) of pBridge (Clontech). The pBridge.Nef. σ 2 plasmid was the same as that used for the Y3H experiments, and alternative versions of this vector (expressing the Nef G2A, WL57,58AA, EEEE62-65AAAA, PP72,75AA, LL164,165AA, and DD174,175AA mutant forms) have been described before (12, 40). To generate the pAD series of vectors, the pMET25-MCS2-PGKT cassette was excised from the pBridge vector by ApaI digestion, polished with DNA polymerase I (New England Biolabs, Ipswich, MA), and inserted into the PvuII site of pGAD424 (Clontech) by blunt-end ligation. This process resulted in a new vector, hereinafter referred to as pAD, which has two MCSs: one downstream of the ADH1 promoter and the GAL4AD sequence (MCS1) and one downstream of the independently controlled MET25 promoter (MCS2). The proteins encoded by genes inserted into these MCSs are both targeted to the yeast nucleus by the presence of nuclear localization signals. The cytosolic tail of human CD4 was amplified by PCR, digested with EcoRI and Sall, and ligated into the corresponding sites of pAD MCS1 to generate pAD.CD4. To create pAD.CD4. α , the α C ORF was amplified by PCR from pGADT7. α by using primers containing NotI and BamHI restriction sites, digested with the appropriate enzymes, and ligated into the NotI-BglII site of pAD.CD4 MCS2. The pAD.CD4. α KR297,340EE plasmid was prepared by a similar procedure, using pGADT7. α KR297,340EE as the PCR template. Assays using these plasmids (including yeast two-hybrid [Y2H], Y3H, and yeast four-hybrid [Y4H] analyses) were carried out according to the Y3H protocol

described above, with only one modification: following the selection of positive transformants, the densities of the corresponding yeast suspensions were normalized to 1.6 OD units (at 600 nm), and the suspensions were then serially diluted to 0.1 OD units before transfer to the +His and –His dropout agar plates. Each Y4H experiment was performed a minimum of three times.

Recombinant protein expression and GST pull-down experiments. The plasmid pST.AP-2 core (expressing residues 1 to 621 of rat α C-adaptin [α trunk] with a C-terminal fusion to glutathione S-transferase [GST], residues 1 to 591 of rat β 2-adaptin [β 2 trunk] with an N-terminal hexahistidine [His_6] tag, residues 1 to 141 of rat μ 2-adaptin [μ 2-N], and residues 1 to 142 of rat σ 2-adaptin [full-length σ 2]) has been described previously (12, 40). pST.AP-2 core α KR297,340EE was generated by site-directed mutagenesis. AP-2 core protein complexes were expressed in *Escherichia coli* BL21(DE3) cells (Novagen, San Diego, CA) and purified as described before (12, 40). To compare the abilities of wild-type α and KR297,340EE mutant α (α KR297,340EE) to associate with the other AP-2 core subunits, the two GST fusion proteins were isolated from bacterial lysates by binding to glutathione-Sepharose beads and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). His_6 -tagged Nef was expressed from the pHIS-Parallel2-Nef vector and purified as described previously (12, 40). The interaction of His_6 -Nef with the wild-type AP-2 core and AP-2 core α KR297,340EE was analyzed by GST pull down (12, 40). Briefly, saturating amounts ($\sim 5 \mu\text{g}$) of the wild-type and mutant AP-2 core complexes were immobilized onto 30 μl of glutathione-Sepharose beads, and the beads were incubated with 3 μg of His_6 -Nef for 2 h at 4°C in a final volume of 1 ml of binding buffer (15 mM HEPES [pH 7.0], 75 mM NaCl, 0.25% [vol/vol] Triton X-100, 0.15% [wt/vol] bovine serum albumin) supplemented with a protease inhibitor cocktail (EDTA-free Complete tablets; Roche Applied Science, Indianapolis, IN). After incubation, the beads were washed three times by resuspension in 1.3 ml of binding buffer lacking bovine serum albumin, followed by centrifugation for 2 min at $2,000 \times g$ and 4°C. Proteins bound to the beads were eluted by resuspension in 50 μl of $2\times$ Laemmli buffer and incubation for 10 min at 90°C. The eluted samples were separated from the beads by centrifugation for 2 min at $16,000 \times g$ and subjected to SDS-PAGE and immunoblotting.

Cells and tissue culture. HeLa cells (American Type Culture Collection, Manassas, VA) were cultured under a humidified atmosphere (5% CO_2) in Dulbecco's modified Eagle medium and passaged every 3 to 4 days. The medium was supplemented with 100 U/ml penicillin, 0.1 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, and 10% (vol/vol) fetal bovine serum.

RNA interference (RNAi) and RNAi-resistant reagents. To simultaneously silence endogenous α A and α C expression in HeLa cells, we designed a single small interfering RNA (siRNA) duplex (sense sequence, 5' GAGCAUGUGCA CGCUGGCCATT; Qiagen, Valencia, CA) targeting nucleotides 1053 to 1072 in the human α A and α C ORFs. An siRNA-resistant version of α C-adaptin (hereinafter referred to as α R) was generated by introducing three silent substitutions into the rat α C cDNA (C $\rightarrow$ T, C $\rightarrow$ T, and G $\rightarrow$ C at nucleotides 1053, 1059, and 1065, respectively, of the rat α C sequence). These substitutions resulted in a total of four mismatches between α R and the siRNA-sensitive human α A/ α C sequences (the rat α C cDNA contains an additional A $\rightarrow$ G substitution at position 1062 compared to the human α A/ α C sequences). The sequence for a V5 epitope tag was fused to the 3' end of the α R ORF, and the α R-V5 cassette was subcloned into the Sall-BamHI sites of pIRES2.EGFP (hereinafter referred to as pIRES.GFP; Clontech) to generate p α R-V5.IRES.GFP. Site-directed mutagenesis of p α R-V5.IRES.GFP yielded p α R-KR297,340EE-V5.IRES.GFP. The presence of an internal ribosome entry site in these plasmids allowed for the independent translation of α R-V5 (or the KR297,340EE mutant form of α R [α R-KR297,340EE] fused to V5) and enhanced green fluorescent protein (GFP) from the same bicistronic transcript.

α -Adaptin RNAi and rescue assays. In HeLa cells, the expression of endogenous α -adaptin was silenced by transfection with siRNA and the endogenous α -adaptin was replaced with α R according to a 7-day protocol. On day 1, HeLa cells grown on six-well plates ($\sim 15\%$ confluence) were either left untreated or transfected with siRNA (100 nM) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and Opti-MEM I (Invitrogen). On day 3, the cells were split at a 1:3 ratio onto fresh six-well plates. On day 4, the cells (at $\sim 50\%$ confluence) were transfected with a second round of siRNA and/or a unique combination of DNA plasmids (pCMV.CD4; pCI or pCI.Nef; and pIRES.GFP, p α R-V5.IRES.GFP, or p α R-KR297,340EE-V5.IRES.GFP) (Table 1) by using Lipofectamine 2000 and Opti-MEM I. The plasmids pCI (empty vector) and pCI.Nef and the pCMV.CD4 plasmid (kindly provided by Klaus Strebel, NIAID, NIH) have been described previously (12, 40). On day 6, the cells were split again onto fresh six-well plates at a 1:2 ratio. On day 7, the cells were detached with 0.5 ml of 2 mM EDTA in phosphate-buffered saline, harvested, and either stained with fluorescent antibodies ($\sim 80\%$ of the cell suspension) or lysed for immunoblot-

TABLE 1. Combinations of siRNA and DNA used for transfection of HeLa cells in AP-2 α rescue assays^a

Row	siRNA	DNA 1	DNA 2	DNA 3
1	None	pIRES.GFP	pCI	pCMV.CD4
2	None	pIRES.GFP	pCI.Nef	pCMV.CD4
3	α -Adaptin	pIRES.GFP	pCI	pCMV.CD4
4	α -Adaptin	pIRES.GFP	pCI.Nef	pCMV.CD4
5	α -Adaptin	paR-V5.IRES.GFP	pCI	pCMV.CD4
6	α -Adaptin	paR-V5.IRES.GFP	pCI.Nef	pCMV.CD4
7	α -Adaptin	paR-KR297,340EE-V5.IRES.GFP	pCI	pCMV.CD4
8	α -Adaptin	paR-KR297,340EE-V5.IRES.GFP	pCI.Nef	pCMV.CD4

^a The various combinations of siRNA duplexes and DNA plasmids used to transfect HeLa cells on day 4 of the α -adaptin RNAi and rescue assays are shown. For each condition, 0.4 μ g of DNA 1, 0.3 μ g of DNA 2, and 0.3 μ g of DNA 3 were used. Cells corresponding to rows 3 to 8 were previously treated with siRNA targeting α -adaptin on day 1 of the assay.

ting (~20%). All silencing and rescue experiments were repeated a minimum of three times.

Antibodies. Allophycocyanin (APC)-conjugated mouse monoclonal antibody to human CD4 (Caltag, Burlingame, CA) and phycoerythrin (PE)-conjugated mouse monoclonal antibody to human CD71 (transferrin receptor [TfR]) (Sigma-Aldrich, St. Louis, MO) were used for fluorescence-activated cell sorter (FACS) analysis. Unconjugated rabbit anti-HIV-1 Nef (NIH AIDS Research and Reference Reagent Program; originally deposited by Ronald Swanson) (70), mouse anti- α -adaptin clone numbers 100/2 (Sigma-Aldrich) and 8/Adaptin α (BD Biosciences, Franklin Lakes, NJ), mouse anti-V5 (Invitrogen), mouse anti- α -tubulin (Sigma-Aldrich), and horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ) were used for immunoblotting.

FACS analysis. HeLa cells stained with the appropriate antibodies were prepared for FACS analysis as described previously (12, 33, 40). Untransfected and unstained HeLa cells were used as a negative control for antibody labeling. GFP fluorescence expressed from the pIRES.GFP series of plasmids was used to identify and gate around transfected cells. The amount of cell-associated APC and PE fluorescence in GFP-positive cells was quantified by a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences).

Immunoblotting. HeLa cells were lysed for 30 min at 4°C in lysis buffer (1% [vol/vol] NP-40 in phosphate-buffered saline) supplemented with a protease inhibitor cocktail (Roche Applied Science). The lysates were then centrifuged for 15 min at 16,000 $\times$ g and 4°C, and the supernatants were stored at -70°C. SDS-PAGE, transfer onto nitrocellulose membranes, labeling with primary antibodies, and detection with secondary antibodies were performed as described previously (12, 40).

RESULTS

Rationale. The Nef flexible loop contains two conserved sequence motifs, a dileucine motif (i.e., ENTSL in NL4-3, EXXXLL consensus for all HIV-1 variants, where X is any amino acid) and a diacidic motif (i.e., DD in NL4-3, (D/E)D consensus for all variants), both of which are required for binding to AP-2 (12, 20, 40, 49) and for CD4 downregulation (2, 10, 12, 16, 26, 33, 40). The dileucine motif, but not the diacidic motif, also mediates the binding of Nef to the homologous AP-1 (γ - β 1- μ 1- σ 1) and AP-3 (δ - β 3- μ 3- σ 3) complexes (33, 40). The Nef dileucine motif belongs to the class of dileucine-based (D/E)XXXL(L/I) sorting signals that are present in many host cell proteins (e.g., the melanosomal enzyme tyrosinase) and that interact with AP-1, AP-2, and/or AP-3 (9). Unlike Nef, other proteins that have (D/E)XXXL(L/I) class signals do not require an additional diacidic motif for binding to AP-2 or the other AP complexes (40). The importance of this diacidic motif in the recognition of Nef by

AP-2 explains the binding sensitivity to high concentrations of salt and indicates that electrostatic forces are critical determinants of this interaction (40). The unique requirement of the Nef diacidic motif for interaction with AP-2 makes it a potential target for specific inhibition of Nef action without interference with the sorting of host cell proteins mediated by dileucine-based signals. These considerations prompted us to map the binding site for the Nef diacidic motif on AP-2 and examine its role in promoting the assembly of a putative CD4-Nef-AP-2 complex.

Identification of basic residues on the AP-2 α subunit that are required for interaction with HIV-1 Nef. Based on the above-described considerations, we hypothesized that the Nef diacidic motif must interact with basic residues on AP-2 that are not conserved in the other two AP complexes. We searched for such residues by comparing the amino acid sequences of the subunits that make up the AP-1 γ - σ 1, AP-2 α - σ 2, and AP-3 δ - σ 3 hemicomplexes, which contain the binding sites for Nef (12, 20, 33, 40, 49). In this way, we identified 21 arginine and lysine residues on α - σ 2 that are not present on the other two hemicomplexes (Fig. 1). We then mutated these residues to either glutamate or aspartate and assayed for the loss of binding of α - σ 2 to wild-type Nef using the Y3H system (12, 40, 49) (Fig. 2A). Several mutations of the α - σ 2 hemicomplex, including a triple mutation of α residues K295, K297, and K298 (initially mutated en bloc because of the close proximity of these residues) to three glutamate residues (yielding the α KKK295,297,298EEE construct) (Fig. 2B), a single mutation of α residue R340 to glutamate (yielding the α R340E construct) (Fig. 2B, see both -His and -His + 3AT rows), and a double mutation of σ 2 residues R124 and K130 to two glutamate residues (yielding the σ 2 RK124,130EE construct), impaired the binding of Nef (Fig. 2C). To determine whether this loss of binding was due to the disruption of the Nef binding site or to more global effects on the hemicomplex, we also tested the α - σ 2 mutant constructs for their abilities to bind the cytosolic tail of mouse tyrosinase. The tyrosinase tail interacts with α - σ 2 in a dileucine-dependent manner that, unlike Nef, does not require a diacidic motif (40). We found that the α KKK295,297,298EEE and α R340E constructs bound to the tyrosinase tail with relatively strong avidities (Fig. 2B), indicating that the mutation of the corresponding residues specifically interfered with the interaction between Nef and AP-2. In contrast, the σ 2 RK124,130EE mutant failed to bind to the tyrosinase tail (Fig. 2C), consistent with an adverse effect of these substitutions on either the folding of the σ 2 subunit or the stability of the α - σ 2 hemicomplex. The remaining mutants, all of which were generated by mutation of the α subunit, interacted equally with Nef and the tyrosinase tail (Fig. 2B and C). To determine whether the interactions detected in the Y3H system were genuine, we tested each of the α mutants for self-activation by substituting σ 1 for σ 2 in the Y3H assay. Given that α and σ 1 do not interact with each other (58), this pair should not be able to bind either Nef or the tyrosinase tail and should not be able to stimulate yeast growth on selective media. Indeed, when combined with σ 1, none of the α mutants showed evidence of interaction with Nef or the tyrosinase tail (Fig. 2B), indicating that (i) the mutations do not cause self-activation, (ii) the observed interactions between the mutant α - σ 2 hemicomplexes and the ligands are genuine, and (iii) the

FIG. 2. Y3H analysis of the interaction of HIV-1 Nef with AP-2 α - σ 2 hemicomplexes having substitutions for nonconserved basic residues. AP-2 α and σ 2 basic residues that are not conserved in the homologous subunits of AP-1 (γ and σ 1) and AP-3 (δ and σ 3) (Fig. 1) were mutated, individually or in combination, to glutamate or aspartate. The resulting α - σ 2 constructs were tested for interaction with HIV-1 Nef or the mouse tyrosinase cytosolic tail by using a Y3H assay, as described in Materials and Methods. (A) Schematic representation of the plasmids used in the Y3H assay. Nef or the tyrosinase cytosolic tail was expressed as a GAL4BD fusion from MCS1 of pBridge, σ 2 was expressed from MCS2 of pBridge, and α was expressed as a GAL4AD fusion from the MCS of pGADT7. Cotransformations of yeast cells with Nef or the tyrosinase tail and discordant combinations of AP-2 α and AP-1 σ 1 constructs were used as self-activation/specificity controls. (B and C) Y3H assay results. Cotransformants were plated onto medium lacking Leu, Trp, Met, and His ($-$ His) in the absence or presence of 1 mM 3AT ($-$ His + 3AT) to detect interaction through *HIS3* reporter gene activation. 3AT is a competitive inhibitor of the His3 protein (imidazoleglycerol-phosphate dehydratase) that increases the stringency of the assay. Cotransformants were also plated onto medium lacking Leu, Trp, and Met (+His) to control for growth and loading. Growth on $-$ His or $-$ His + 3AT plates is indicative of interactions. WT, wild type.

FIG. 3. AP-2 α residues K297 and R340 contribute to the interaction between α - σ 2 and HIV-1 Nef. The effects of single or combined mutations of AP-2 α K295, K297, K298, and/or R340 to glutamate or alanine on the interaction of AP-2 α - σ 2 with HIV-1 Nef or the mouse tyrosinase cytosolic tail were investigated. The discordant α and σ 1 pair was used as a negative control. Analyses were performed using the Y3H system as described in the legend to Fig. 2 and in Materials and Methods.

transmembrane proteins from the plasma membrane (31, 32, 46, 50, 51). FACS analysis showed that the depletion of endogenous α -adaptin increased the surface levels of one such transmembrane protein, TfR (Fig. 6A, left panel), in agreement with previous findings (31, 32, 51). This result was consistent with the presence of an endocytic, tyrosine-based sorting signal (i.e., YTRF) in the TfR cytosolic tail (14, 47) that interacts with the μ 2 subunit of AP-2 (53, 56). Interestingly, α -adaptin depletion also increased the surface levels of CD4 (Fig. 6A, right panel), probably by reducing the basal rate of CD4 endocytosis mediated by a dileucine-based sorting signal (i.e., SQIKRLL) in the CD4 cytosolic tail (1, 61). We then tested for the rescue of AP-2 function in α -adaptin-depleted cells by transfection with RNAi-resistant versions of wild-type or KR297,340EE mutant α -adaptin. We found that both constructs reduced surface TfR (Fig. 6B, left panel) and CD4 (Fig. 6B, right panel) to approximately normal levels, indicating that α K297 and R340 are not required for endocytosis mediated by tyrosine- and dileucine-based sorting signals. We next used the RNAi-rescue assay with HeLa cells expressing CD4 with or without Nef. The depletion of α -adaptin completely eliminated the ability of Nef to downregulate CD4 from the cell surface (Fig. 6C). Importantly, the expression of the wild-type α R construct in α -depleted cells restored the ability of Nef to downregulate CD4 (Fig. 6D, left panel), whereas the expression of the KR297,340EE mutant α construct did not (Fig. 6D, right panel). Immunoblot analysis of the transfected cells indicated that this disparity in Nef function was not due to

differences in the silencing of endogenous α , the expression of α R constructs, or the expression of Nef itself (Fig. 6E). These analyses thus demonstrated that α -adaptin residues K297 and R340 are specifically required for Nef-induced downregulation of CD4.

Requirement of AP-2 α -adaptin residues K297 and R340 for cooperative assembly of a tripartite Nef-CD4-AP-2 complex.

Nef is believed to downregulate CD4 by binding to the cytosolic tail of this receptor and linking it to AP-2, thereby accelerating the rate of CD4 endocytosis. Although previous work has provided evidence for weak binary interactions between Nef and CD4 (7, 28, 62, 67), Nef and AP-2 (12, 40), and CD4 and AP-2 (30), the occurrence of a tripartite complex involving all three components has not yet been demonstrated. We analyzed the possible formation of such a complex by using a combination of Y2H, Y3H, and Y4H assays. Yeast were transformed with the pBridge and pAD vectors, each of which contained two MCSs under the control of independent promoters (Fig. 7A). In this way, we were able to express up to four proteins, each of which was targeted to the nucleus by nuclear localization signals. We tested for the interaction between Nef (expressed as a GAL4BD fusion protein) and the cytosolic tail of CD4 (expressed as a GAL4AD fusion protein) either alone or in the presence of the AP-2 α and σ 2 subunits. We observed that, in the absence of α and σ 2, Nef and CD4 did not detectably interact in our system (Fig. 7B). This result differed from previous Y2H findings (67), probably because of the greater stringency of our assay. Moreover, individual ex-

FIG. 4. Location of AP-2 α K297 and R340 on the three-dimensional structure of the AP-2 core complex (accession no. 1GW5 and 2VGL) (15). (A and B) Surface representations with the α , β 2, μ 2, and σ 2 subunits colored in blue, green, magenta, and gold, respectively; the binding site for polyphosphoinositides (PIPs) in the α subunit is depicted in light blue, and the α K297 and R340 residues (including their side chains) are shown in red. The image in panel B corresponds to a 60° rotation around the x axis of that shown in panel A. Residues α K297 and R340 correspond to K298 and R341 in polymer 1 of the crystal structure (15). (C) Surface representation of the image in panel A colored according to electrostatic potential (contoured as red to blue from -74 to 74 kT). (D) Magnified ribbon diagram of the region surrounding residues K297 and R340. All images were drawn with PyMOL (17).

pression of either α or σ 2 failed to promote interaction between Nef and CD4 (Fig. 7B). However, when both α and σ 2 were expressed, Nef bound to the CD4 cytosolic tail (Fig. 7B). The increased avidity of CD4 for Nef in the presence of the α - σ 2 hemicomplex indicates that a CD4-Nef- α - σ 2 complex is formed by cooperative assembly. To test whether the assembly of this complex is dependent on the known binary interactions between its components, we analyzed several mutants by using a Y4H assay (Fig. 7C). Mutations that inhibit the binding of Nef to either CD4 (Nef WL57,58AA) (28) or AP-2 (Nef LL164,165AA and DD174,175AA) (12, 20, 40) prevented the formation of the tripartite complex (Fig. 7C). Significantly for this study, the mutation of α -adaptin residues K297 and R340, which are required for the binding of AP-2 to Nef (Fig. 3 and 5), yielded the same result (Fig. 7C). In contrast, mutations of three Nef motifs that are not required for binding to either CD4 or AP-2 (Nef G2A, EEEE62-65AAAA, and PP72,75AA) (28, 62) did not significantly affect the formation of the complex. Thus, the assembly of a CD4-Nef-AP-2 complex depends

on the known binary interactions between its individual components, verifying the specificity of the assays.

DISCUSSION

Our search for a binding site for the Nef diacidic motif on AP-2 has identified two basic residues, K297 and R340, on the surface of the α -adaptin subunit that are specifically required for both Nef binding (Fig. 2, 3, and 5) and CD4 downregulation (Fig. 6). In contrast, these basic residues are dispensable for binding to the cytosolic tail of tyrosinase (Fig. 2 and 3), which is entirely dependent on a dileucine-based sorting signal (40). Moreover, the mutation of these basic residues does not affect the ability of AP-2 to maintain steady-state levels of TfR and CD4 at the cell surface (Fig. 6), which is dependent on endocytosis mediated by tyrosine-based (14, 47, 53, 56) and dileucine-based (1, 30, 61) signals, respectively. Finally, these basic residues are not conserved in the homologous AP-1 γ and AP-3 δ subunits, which, in combination with the corresponding

FIG. 5. The replacement of α K297 and R340 impairs the binding of the recombinant AP-2 core to Nef in vitro. (A) Recombinant Nef and AP-2 core constructs used in the in vitro binding experiments were produced as described in Materials and Methods and subjected to SDS-PAGE on 4 to 12% acrylamide gradient gels, followed by Coomassie blue staining. Lanes corresponding to the AP-2 core complexes bearing wild-type (WT) and KR297,340EE mutant α chains show the α trunk-GST, His₆- β 2 trunk, μ 2-N, and full-length σ 2 subunits in order of increasing mobilities (μ 2-N and σ 2 comigrate in this gel system). The band at ~21 kDa (asterisk) in the lanes corresponding to wild-type and mutant AP-2 cores represents a GST degradation product. (B) Equal amounts of GST fusion proteins with wild-type (WT) or α KR297,340EE-containing AP-2 cores, or the ear domains of AP-2 α C (57) or AP-3 β 3B (18) (the latter two as negative controls), were immobilized on glutathione-Sepharose beads, and the beads were incubated with recombinant His₆-Nef for 2 h at 4°C. Bound proteins were eluted and subjected to SDS-PAGE on 10% acrylamide gels, followed by immunoblotting (IB) with antibodies to the His₆ tag (upper panel) or to Nef (lower panel). The last lane on the right (labeled -) shows binding to unfused GST. The ~60-kDa band labeled by anti-His₆ in the upper blot corresponds to the His₆-tagged β 2 trunk in the recombinant AP-2 core and served as an internal loading control. Numbers to the left indicate the positions of molecular mass markers (in kilodaltons).

σ 1 and σ 3 subunits, also bind dileucine-based sorting signals (12, 20, 33, 40). Thus, Nef appears to exploit a unique feature of AP-2 in order to engage the endocytic machinery. Like the Nef diacidic motif, the α -adaptin site comprising K297 and R340 is therefore a potential target for pharmacologic interference with Nef-induced CD4 downregulation (21).

Characteristics of the α -adaptin basic patch containing K297 and R340. Residues K297 and R340 on α -adaptin are part of a large basic patch that rivals in size the binding site for polyphosphoinositides near the N terminus of the protein (15) (Fig. 4C). K297 is contained within a loop that also comprises α -adaptin residues K295 and K298 (Fig. 1A) (15). Although the individual mutation of K295 or K298 did not affect Nef binding in the Y3H assay (Fig. 3), we cannot rule out the possibility that the double mutation of those residues may have some effect. It is notable that AP-1 γ and AP-3 δ lack not only residues homologous to K297 and R340 but also a basic loop like that containing K295, K297, and K298 in α -adaptin (Fig. 1A). Remarkably, all of these basic residues are conserved on both the α C and α A mammalian isoforms, as well as in α -adaptins from a wide range of metazoan species, including the genetic model organisms *Drosophila melanogaster* and *Cae-*

norhabditis elegans (see Fig. S1 in the supplemental material). Since Nef-encoding immunodeficiency viruses infect only primates, this phylogenetic conservation hints at a more general function of these basic residues. Perhaps this site is involved in the recognition of clusters of acidic residues, either alone or in combination with other sorting signals. The cytosolic tail of the mammalian endopeptidase furin has a stretch of acidic residues interspersed with serine residues (i.e., SDSEEDE) that are substrates for casein kinase II-dependent phosphorylation. Together with sequences resembling tyrosine-based and dileucine-based sorting signals, this acidic cluster contributes to the localization of furin to the trans-Golgi network (35, 68, 78) and to its sorting to the basolateral plasma membrane domain of polarized epithelial cells (71). Remarkably, the acidic cluster is intrinsically capable of mediating clathrin-dependent endocytosis in the absence of any other signal (43, 78). Similar acidic clusters are found in the cytosolic tails of furin homologs from all metazoans. Although the phosphorylated mammalian furin cluster has been shown previously to bind a protein named PACS-1 (79), in light of our results, it would be of interest to test whether this cluster also mediates binding to AP-2 via the basic patch described here.

Possible location of the dileucine binding site on AP-2. The mutational approach used here for identifying the binding site for the Nef diacidic motif could also be applied to the mapping of the binding site for the Nef dileucine motif or other dileucine-based sorting signals on AP-2. In fact, the demonstration that α -adaptin residues K297 and R340 are required for Nef binding constrains the area of α - σ 2 that is likely to contain the dileucine binding site. The Nef dileucine motif (i.e., ENTSL in NL4-3 and consensus sequence EXXXLL in all HIV-1 variants) comprises not only L164 and L165 but also E160, which is partially required for AP-2 binding (12). The two residues that make up the Nef diacidic motif, D174 and D175, lie 14 and 15 residues, respectively, in the C-terminal direction from E160 along the Nef flexible loop. Assuming a distance of 3.8 Å between contiguous α -carbon atoms in a linear peptide chain, E160 would be at most 53 Å from D174 and 57 Å from D175. Based on these considerations, we scanned the three-dimensional structure of AP-2 (15) for basic residues that are located up to 57 Å from α -adaptin residues K297 and R340 and that are conserved in AP-1 and AP-3 (since these complexes also bind the Nef dileucine motif). Within this range are two residues in close proximity to each other, α R21 and σ 2 R15, the mutation of which impairs the binding of both the Nef and tyrosinase dileucine motifs (R. Chaudhuri, unpublished observations). Nestled between the two regions of AP-2 that contain α R21 and σ 2 R15 and α K297 and R340 (Fig. 4A and B) are several σ 2 residues where the binding site for the dileucine pair may be located.

While this paper was under revision, Kelly et al. reported the crystal structure of AP-2 in a complex with a variant dileucine-based signal from CD4 (36). The CD4 signal differs from typical (D/E)XXXL(L/I) signals in that a phosphoserine-glutamine pair substitutes for the acidic residue in the canonical motif (9). The crystal structure indicates that the phosphoserine-glutamine moiety likely binds to the α R21 and σ 2 R15 residues and that the two leucines bind to adjacent pockets lined by several hydrophobic σ 2 residues, including V88 and

L103 (36). These findings are consistent with the results of our mutational analysis and explain why both α and $\sigma 2$ are required for interactions with dileucine-based signals in Y3H assays (12, 40) and in vitro binding assays (20). We hypothesize that the Nef dileucine motif binds to the same site on α - $\sigma 2$ as the CD4 dileucine signal and that the unique Nef diacidic motif extends the interaction to the distal part of α , conferring greater affinity and specificity.

Cooperative assembly of a tripartite complex comprising the CD4 tail, Nef, and AP-2. To our knowledge, the Y4H assay results reported here represent the first demonstration of the occurrence of a tripartite complex comprising the CD4 tail, Nef, and AP-2. The detection of this complex is dependent on the known determinants of bimolecular interactions between CD4 and Nef (i.e., Nef residues W57 and L58 that bind to the CD4 tail) and between Nef and AP-2 (i.e., the Nef dileucine and diacidic motifs and α -adaptin residues K297 and R340) (Fig. 7). Furthermore, its detection is independent of Nef residues that do not participate in binding to CD4 or AP-2, such as the myristoylation site (G2), the acidic cluster (EEEE62-65), and the polyproline motif (comprising P72 and P75) (Fig. 7). Importantly, these results correlate with previously published functional data: mutations that disrupt the formation of the CD4-Nef-AP-2 complex also prevent the downregulation of CD4 from the plasma membrane (1, 2, 12, 40, 73, 75; this study), while mutations that do not affect the assembly of the complex are not essential for CD4 downregulation (1, 12, 44, 73). The only exception to this correlation is the Nef G2A mutant, which fails to downregulate CD4 from the cell surface because it is not myristoylated and therefore cannot efficiently associate with the plasma membrane (7, 73, 81). However, myristoylation of Nef is not necessary for its incorporation into the CD4-Nef-AP-2 complex in the Y4H assay, as all the proteins in this complex are targeted to the yeast nucleus by the presence of heterologous nuclear localization signals. Given the requirement of the dileucine motif in the CD4 cytosolic tail for both constitutive and Nef-induced CD4 downregulation (1, 64), it will now be important to examine the role of this motif

in the formation of the CD4-Nef-AP-2 complex. Another acidic residue on Nef, D123, has also been shown previously to be required for CD4 downregulation (42, 55), and it would be of interest to test if this requirement reflects a role in the assembly of the CD4-Nef-AP-2 complex or, as previously proposed, in Nef oligomerization (4) or interaction with another host protein (13).

The assembly of a CD4-Nef-AP-2 complex is consistent with the previous proposal that Nef links the tail of CD4 to AP-2 (54, 60). In addition, our data show that the α - $\sigma 2$ hemicomplex promotes the interaction of the CD4 tail with Nef (Fig. 7). Thus, the formation of the CD4-Nef-AP-2 complex involves cooperative interactions among all of its components. A possible explanation for this cooperativity is that the binding of AP-2 to one site on Nef increases the affinity of a second site on Nef for the CD4 tail, a phenomenon that is formally known as positive heterotropic cooperativity (69). In this model, AP-2 does not make direct contact with the CD4 tail but causes a conformational change in Nef that enhances binding to the CD4 tail. An alternative explanation is that each component of the complex makes simultaneous contacts with the other two, thereby enhancing the overall stability of the tripartite complex. This model requires that both Nef and AP-2 bind to the CD4 tail directly and at the same time. Further biochemical and structural analyses will be needed to distinguish between these models and to identify all the determinants of assembly of the CD4-Nef-AP-2 complex.

In T cells, CD4 exists in a complex with the protein tyrosine kinase Lck (77; reviewed in reference 54). In this complex, the CD4 cytosolic tail and the N terminus of Lck form a folded zinc clasp structure (37). The engagement of the CD4 tail by Nef likely requires (or induces) the dissociation of Lck (25), with subsequent unfolding of the CD4 tail (37). The assembly of a stable CD4-Nef-AP-2 complex may preclude the reassociation of Lck with CD4, thereby promoting the internalization and eventual lysosomal degradation of CD4 (64).

Nef has also been shown previously to engage in cooperative interactions with other receptors and AP complexes. For ex-

FIG. 6. AP-2 α residues K297 and R340 are required for Nef-induced CD4 downregulation. HeLa cells were subjected to a 7-day siRNA-cDNA transfection protocol as described in Materials and Methods and in Table 1. Control and α siRNA-treated cells were cotransfected with three plasmids: one expressing CD4, one lacking (–) or expressing (+) Nef, and one lacking or expressing either wild-type α R (α R-WT) or α R-KR297,340EE. The cells were then prepared for FACS analysis and immunoblotting. Cells prepared for FACS analysis were either left unlabeled as a control for background fluorescence (shaded curves in all plots) or stained with PE-conjugated anti-human TfR or APC-conjugated anti-human CD4 antibodies. (A) Treatment with α siRNAs increases cell surface TfR and CD4 levels in the absence of Nef. (B) Both wild-type α R and α R-KR297,340EE prevent the increase in the levels of TfR and CD4 caused by treatment with α siRNA in the absence of Nef. (C) Nef expression downregulates CD4 in control but not α siRNA-treated HeLa cells. (D) Rescue of Nef-induced downregulation of CD4 by wild-type α R but not α R-KR297,340EE in α siRNA-treated HeLa cells. The results shown are representative of data from three independent experiments with similar results. Statistical analyses of the data from these three experiments showed that Nef-dependent CD4 downregulation (expressed as the ratio of geometric means in the absence or presence of Nef) was 4.81 ± 0.70 for control cells, $1.51 \pm 0.24^*$ for α siRNA-treated cells, 4.60 ± 0.94 for α siRNA-treated cells expressing wild-type α R, and $1.50 \pm 0.04^{*\dagger}$ for α siRNA-treated cells expressing α R-KR297,340EE (mean $\pm$ standard error of the mean; $n = 3$), with the symbols * and † indicating values that are significantly different ($P < 0.05$) from those for control cells and α siRNA-treated cells expressing wild-type α R, respectively, as calculated by analysis of variance followed by two-tail Dunnett's test. (E) Aliquots of the transfected cells from all experimental groups were lysed and subjected to SDS-PAGE and immunoblotting (IB) with the antibodies indicated to the right. All cells were transfected with a plasmid encoding CD4, together with the plasmids and oligonucleotides indicated in the grid above the lanes (+, present; –, absent; KREE, KR297,340EE mutant). Notice that the anti-AP-2 α clone 100/2 recognized both endogenous isoforms of α -adaptin, α A and α C (apparent as an ~ 100 -kDa doublet in which the upper and lower bands represent α A and α C, respectively) (5), as well as a nonspecific band (~ 85 kDa). The anti-AP-2 α clone 8/Adaptin α , however, recognized only endogenous α A-adaptin since it was raised against a fragment of this isoform. The V5-tagged, RNAi-resistant α C rescue constructs were detected by both the 100/2 anti- α antibody and the anti-V5 antibody. Immunoblotting with anti- α -tubulin was used as a loading control. Numbers to the left indicate the positions of molecular mass markers (in kilodaltons).

FIG. 7. Assembly of a tripartite complex of Nef, the cytosolic tail of CD4, and the AP-2 α - σ 2 hemicomplex as demonstrated by yeast hybrid assays. (A) Plasmids used in the Y2H, Y3H, and Y4H assays. In all assays, HIV-1 Nef was expressed from pBridge as a GAL4BD fusion protein, while the cytosolic tail of human CD4 (CD4 ct) was expressed from pAD as a GAL4AD fusion protein. In the Y2H assay, no other proteins were expressed from these vectors; in the Y3H assay, either σ 2-adaptin or α C-adaptin was expressed from pBridge or pAD, respectively; in the Y4H assay, both σ 2 adaptin and α C adaptin were coexpressed. (B) Y2H, Y3H, and Y4H analyses of the interaction between GAL4BD-Nef and GAL4AD-CD4 in the absence or presence of one or both components of the α - σ 2 hemicomplex. The plasmids used in the yeast hybrid experiments are noted to the left of the panel, with the pBridge (pBr)-based vectors in the first column and the pAD-based vectors in the second column. Row 1 corresponds to the Y2H assay, rows 2 and 3 correspond to the Y3H assays, and row 4 corresponds to the Y4H assay. Yeast from all assays were seeded onto +His and -His plates at increasing levels of OD. Yeast growth on the -His plates indicates an interaction between GAL4BD-Nef and GAL4AD-CD4. (C) Y4H analysis of the effect of several Nef and α mutants on the interaction of GAL4BD-Nef and GAL4AD-CD4 in the presence of the α - σ 2 hemicomplex.

ample, the binding of HIV-1 Nef to the cytosolic tail of MHC-I increases the affinity of a tyrosine-based motif in this tail for the μ 1 subunit of AP-1, thus stabilizing the assembly of an MHC-I-Nef-AP-1 complex (52, 80). In addition, the binding of

simian immunodeficiency virus Nef to the cytosolic domain of the CD3- ζ chain of the T-cell antigen receptor increases the affinity of this domain for AP-2, leading to the stabilization of a CD3- ζ -Nef-AP-2 complex (74). These cooperative interac-

tions enable Nef to downregulate MHC-I from an intracellular location and the CD3- ζ chain from the cell surface. Together with our findings, these observations indicate that the establishment of cooperative interactions with various host cell proteins is a general property of Nef that underlies its effects on protein trafficking.

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