HIV-1 Nef-induced Down-Regulation of MHC Class I Requires AP-1 and Clathrin but Not PACS-1 and Is Impeded by AP-2[□]

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Major histocompatibility complex class I is down-regulated from the surface of human immunodeficiency virus (HIV)-1-infected cells by Nef, a virally encoded protein that is thought to reroute MHC-I to the *trans*-Golgi network (TGN) in a phosphofurin acidic cluster sorting protein (PACS) 1, adaptor protein (AP)-1, and clathrin-dependent manner. More recently, an alternative model has been proposed, in which Nef uses AP-1 to direct MHC-I to endosomes and lysosomes. Here, we show that knocking down either AP-1 or clathrin with small interfering RNA inhibits the down-regulation of HLA-A2 (an MHC-I isotype) by Nef in HeLa cells. However, knocking down PACS-1 has no effect, not only on Nef-induced down-regulation of HLA-A2 but also on the localization of other proteins containing acidic cluster motifs. Surprisingly, knocking down AP-2 actually enhances Nef activity. Immuno-electron microscopy labeling of Nef-expressing cells indicates that HLA-A2 is rerouted not to the TGN, but to endosomes. In AP-2-depleted cells, more of the HLA-A2 localizes to the inner vesicles of multivesicular bodies. We propose that depleting AP-2 potentiates Nef activity by altering the membrane composition and dynamics of endosomes and causing increased delivery of HLA-A2 to a prelysosomal compartment.

INTRODUCTION

Like many viruses, human immunodeficiency virus (HIV)-1 has evolved strategies to avoid detection and destruction by the host. One such strategy is the down-regulation of major histocompatibility complex (MHC) class I from the plasma membrane of HIV-1-infected cells, which prevents the cells from being attacked by cytotoxic T lymphocytes. MHC-I down-regulation has been shown to be a function of a virally encoded protein called Nef (Schwartz *et al.*, 1996; Collins *et al.*, 1998). Nef is one of the so-called “accessory proteins” of both human and simian immunodeficiency viruses: although not required for viral replication *in vitro*, Nef plays a key role in the development of acquired immunodeficiency syndrome. Nef is a small protein, only ~200 amino acids, but it has been reported to interact with a large number of host cell proteins, including several proteins that are involved in membrane traffic. These interactions are thought to be responsible for the ability of Nef to modulate the

surface expression of MHC-I and other molecules (for review, see Collins and Baltimore, 1999; Piguet *et al.*, 1999; Doms and Trono, 2000; Roeth and Collins, 2006).

Among the binding partners that have been identified for Nef are the adaptor protein (AP) complexes. There are four AP complexes in mammalian cells, two of which, AP-1 and AP-2, are highly enriched in clathrin-coated vesicles (CCVs). AP-1 facilitates clathrin-mediated trafficking between the *trans*-Golgi network (TGN) and endosomes (although there is still some question about directionality), and AP-2 facilitates clathrin-mediated endocytosis (Robinson, 2004). AP-3, which seems to be able to act both in a clathrin-dependent and in a clathrin-independent manner (Dell’Angelica *et al.*, 1998; Peden *et al.*, 2004; Borner *et al.*, 2006), participates in trafficking to lysosomes and lysosome-related organelles (Robinson and Bonifacio, 2001). The function of AP-4 is still unknown. Nef has been shown to bind to the AP complexes via a typical dileucine motif, ENTSL (Janvier *et al.*, 2003; Chaudhuri *et al.*, 2007). However, mutations in this motif do not impair the ability of Nef to down-regulate MHC-I (Greenberg *et al.*, 1998a). In addition, a dominant-negative mutant of dynamin, which blocks both clathrin-mediated and caveolae-mediated endocytosis, has no apparent effect on Nef-induced MHC-I down-regulation, indicating that Nef acts mainly by diverting intracellular MHC-I away from the plasma membrane rather than by promoting the internalization of MHC-I that is already at the plasma membrane (Le Gall *et al.*, 2000; also see Kasper and Collins, 2003).

Mutagenesis studies on Nef have pinpointed a cluster of acidic residues, EEEE (amino acids 62–65), as being important for MHC-I down-regulation (Greenberg *et al.*, 1998b;

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Abbreviations used: AP, adaptor protein; CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; CPD, carboxypeptidase D; LAMP, lysosome-associated membrane protein; MVB, multivesicular body; PACS, phosphofurin acidic cluster sorting protein.

Williams *et al.*, 2005). The current working model is that Nef uses this acidic cluster to bind to phosphofurin acidic cluster sorting protein (PACS) 1 (Doms and Trono, 2000). PACS-1 is a protein that was originally identified in a yeast two-hybrid library screen as a binding partner for furin, an enzyme that cycles between the TGN and endosomes (Wan *et al.*, 1998). Retrieval of furin from endosomes is dependent on a cluster of acidic residues in its cytoplasmic tail, and PACS-1 has been shown to bind *in vitro* to the acidic cluster sequences of both furin and Nef (Piguet *et al.*, 2000). In addition, antisense RNA directed against PACS-1, which causes an eightfold decrease in PACS-1 expression, partially inhibits Nef-induced MHC-I down-regulation (Piguet *et al.*, 2000), and overexpressing a phosphorylation-deficient mutant of PACS-1 has a similar effect (Scott *et al.*, 2003).

PACS-1 has been proposed to act in turn via adaptor complexes, in particular AP-1. Immunoprecipitation of PACS-1 brings down AP-1 but not AP-2, and this interaction has been mapped to a stretch of eight residues. Substituting these amino acids with eight alanines prevents coimmunoprecipitation with AP-1, and overexpression of the alanine-substituted construct has a dominant-negative effect on both furin sorting and Nef-induced MHC-I down-regulation (Crump *et al.*, 2001). Thus, the model is that MHC-I binds to Nef, which binds to PACS-1, which binds to AP-1, which binds to clathrin, and that these interactions bring MHC-I into an endosome-TGN recycling loop and keep it off the plasma membrane (Doms and Trono, 2000).

Because dominant negatives rely on overexpression and can have indirect effects (e.g., by titrating out other binding partners), we decided to test the possible roles of AP-1, clathrin, PACS-1, PACS-2 (a homologue of PACS-1), and AP-2 in Nef-induced down-regulation of MHC-I by carrying out small interfering RNA (siRNA) knockdowns. The cells were treated with the siRNAs, transiently transfected with a Nef-encoding plasmid, and assayed for Nef expression and surface MHC-I by flow cytometry. While this work was in progress, Roeth *et al.* (2004) published a report in which they used siRNA knockdowns to investigate the role of AP-1 in Nef-expressing T cells and astrocytes. They found that depleting AP-1 inhibits the ability of Nef to reduce the amount of MHC-I on the cell surface. In the present study, we demonstrate that knocking down AP-1 has a similar effect in HeLa cells. In addition, we explore the functions of clathrin, PACS-1, and PACS-2 in Nef-induced down-regulation of MHC-I, as well as in the sorting of acidic cluster motifs in general. We also investigate the possible involvement of AP-2, and we find that unexpectedly, knocking down AP-2 dramatically enhances the ability of Nef to down-regulate MHC-I.

MATERIALS AND METHODS

Cell Lines

HeLaM cells (Tiwari *et al.*, 1987) were used as the parental cell line for all of our experiments. The HLA-A2-expressing cell line was generated by Hewitt *et al.* (2002). The CD8-furin_{WT}-expressing HeLa cell line was a kind gift from Matthew Seaman (University of Cambridge; Seaman, 2004). Two mutant HeLa cell lines, expressing CD8-furin_{AKGL} and CD8-furin_{AKGL-AAAA}, were generated for this study. A QuikChange mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce the mutations into the CD8-furin plasmid (Seaman, 2004), and stable cell lines were selected as described previously (Motley *et al.*, 2006). For the comparison between HeLa and T-cells, a viral-specific CD8 T cell line was used (MacAry *et al.*, 2004).

siRNA Knockdowns

Knockdowns were carried out on the CD8-chimera cell lines as described previously (Motley *et al.*, 2003). An amended 6-d protocol was used for knockdowns in HeLa-HLA-A2 cells. The siRNA treatment was carried out as

described above, but cells were transfected with plasmids on day 4 for 24 h followed by a shift to 26°C for a further 24 h on day 5. New siRNAs directed against PACS-1 were synthesized for this study with the following sequences: GACGAAGAUCUCCGGAAAG (PACS-1 siRNA1), AGCAUCCUCAGCAGC-CCAA (PACS-1 siRNA2), GAUCGUCCUCCAGCUAGU (PACS-1 siRNA3), and GCUAGUGGACUGGUGGAAA (PACS-1 siRNA4). We also used a previously published PACS-1 siRNA (Crump *et al.*, 2003). For knocking down AP-3 δ , an siRNA was synthesized with the sequence GGACGAGGCAAAUACAUA (Peden, personal communication). siRNA smartpools KIAA0602 and AP1G1 were used against PACS-2 and AP-1 γ , respectively. Knockdowns of AP-1 μ 1A, AP-2 α , AP-2 μ 2, and clathrin heavy chain were carried out using siRNAs that have been described previously and shown to be effective (Hirst *et al.*, 2003; Motley *et al.*, 2003). All siRNAs were from Dharmacon RNA Technologies (Lafayette, CO), except for the δ siRNA, which was from Ambion (Austin, TX).

Plasmids

Transfections with pIRES plasmids were carried out using Mirus TransIT HeLa MONSTER transfection reagent (Cambridge Bioscience, Cambridge, United Kingdom) for 48 h as described above, and standard immunofluorescence labeling was performed as described previously (Motley *et al.*, 2003). The control (Nef_{STOP}) and wild-type (Nef_{WT}) plasmids have already been described (as pCG.NL43Nef⁺pIRESgfp and pCG.NL43Nef⁺pIRESgfp, respectively; Schindler *et al.*, 2003). The two mutant plasmids (Nef_{LL164-165AA} and Nef_{EEEE62-65AAAA}) were constructed using a QuikChange mutagenesis kit (Stratagene).

Antibodies

Several different monoclonal antibodies against MHC-I were used. For flow cytometry and immunoprecipitation, we used BB7.2 (BD Biosciences, San Jose, CA) (Parham and Brodsky, 1981), a conformation-specific antibody that binds to HLA-A2 and HLA-A28. For Western blotting and immunogold electron microscopy (EM), we used HCA2, a kind gift from Emmanuel Wiertz (University of Leiden; Stam *et al.*, 1990), which preferentially binds HLA-A isotypes. For immunofluorescence, we used W6/32 (Barnstable *et al.*, 1978), which recognizes HLA-A, HLA-B, and HLA-C. When used on HLA-A2-expressing HeLa cells, all of the above-mentioned antibodies exclusively or primarily recognized HLA-A2, because it has been shown that expression of other MHC-I isotypes is greatly reduced when a particular isotype is overexpressed (Joyce, 1997). For flow cytometry on the parental HeLa cell line, which expresses HLA-A6802/B1503/C1203 (Peter Cresswell, personal communication), we used W6/32 (see above) and 4E (Yang *et al.*, 1984; Zinszner *et al.*, 1990), which binds HLA-B and HLA-C.

Other antibodies used in this study were in-house antibodies against μ 3, clathrin heavy chain (CHC) (Borner *et al.*, 2006), and Nef (MATG 020; Stumptner-Culetto *et al.*, 2003); commercially available antibodies against μ 2 and α (both from BD Biosciences Transduction Laboratories, Lexington, KY), γ (mAb100.3; Sigma-Aldrich, St. Louis, MO), and CD8 (153-020; Ancell, Bayport, MN); a polyclonal antibody against AP-3 δ , which was a kind gift from Andrew Peden (University of Cambridge); and a polyclonal antibody against μ 1A, which was a kind gift from Linton Traub (University of Pittsburgh). In-house antibodies were also generated against PACS-1 and PACS-2. Residues 428–493 (for PACS-1) and 348–419 (for PACS-2) were amplified by polymerase chain reaction (PCR) from IMAGE Clones 3091722 and 6371669, respectively, and cloned into pGEX-T1 to generate glutathione S-transferase fusion proteins. Expression and purification of the fusion proteins, the immunization protocol, and affinity purification of the resulting antisera were performed as described previously (Hirst *et al.*, 2000). Secondary antibodies for immunofluorescence and flow cytometry were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were used for Western blotting.

Immunofluorescence and Western Blotting

Immunofluorescence labeling was carried out as described previously (Motley *et al.*, 2003). For triple immunofluorescence labeling, cells were fixed with paraformaldehyde for 20 min, quenched with 0.05M NH₄Cl in phosphate-buffered saline (PBS) for 5 min, and then they were stained with W6/32 followed by Alexa 555-conjugated anti-mouse immunoglobulin. Cells were subsequently permeabilized with 0.1% Triton X-100 for 9 min, and then they were restained with W6/32 followed by Alexa 647-conjugated anti-mouse immunoglobulin before imaging. Cells were viewed using a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a charge-coupled device camera (Princeton Scientific Instruments, Monmouth Junction, NJ), and photographs were recorded using IP Lab software (BD Biosciences, Rockville, MD).

Western blots of whole cell homogenates were carried out as described previously (Borner *et al.*, 2006). HeLa-HLA-A2 and T cell homogenates were matched for total protein concentration before electrophoresis using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Flow Cytometry

Cells were harvested 48 h after transfection using a trypsin-EDTA solution (Sigma-Aldrich). To assay for Nef expression, the cells were then fixed with 2% paraformaldehyde, permeabilized with 1% saponin, and blocked in 10% fetal bovine serum, and then they were labeled with anti-Nef followed by Cy5-conjugated goat anti-mouse immunoglobulin (Invitrogen). To assay for surface HLA-A2, unfixed cells were incubated at 4°C with anti-HLA-A2 (BB7.2) followed by the same secondary antibody, and 7-amino-actinomycin D (7-AAD) (Calbiochem, San Diego, CA) was used to distinguish live cells. The commercially available 9E10 (anti-myc) monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as an antibody control for background labeling. Flow cytometric data were acquired using a two-laser, four-color FACSCalibur flow cytometer (BD Biosciences). Data analysis was done using CELLQuest (BD Biosciences) and FloJo software (Tree Star, Ashland, OR), and the mean of green fluorescent protein (GFP)-positive cells was used to compare and plot data. For electron microscopy and for the Western blotting experiment shown in Figure 7b, the GFP-positive cells were sorted using a MoFlo cell sorter (Dako UK, Ely, Cambridgeshire, United Kingdom) into fresh, chilled medium containing 10% fetal calf serum.

Flow cytometry data were analyzed using FlowJo (version 6.3.4; Tree Star). Cells were identified by their forward scatter/side scatter and 7-AAD profiles, and gates were used to distinguish live cells from debris for further analysis. To control for differences in overall surface MHC-I staining between samples, the mean fluorescence intensity (MFI) for the GFP⁺ and GFP[−] populations was expressed as a ratio (GFP⁺/GFP[−]). Nef function was then calculated (Nef_{WT}/Nef_{STOP}). Transfection efficiency varied between experiments performed on different days; therefore, to allow for direct comparison of these experiments, test samples were standardized to the average for all Nef_{STOP} samples assayed on one particular day. Knockdown and no knockdown samples were then compared, and differences were calculated. The average and SD was calculated for at least three independent repeats of each knockdown. The values plotted relate to the amount of MHC-I retained at the cell surface in each condition; therefore, each value is inversely proportional to Nef activity.

Pulse-Chase Assay

For each time point, 1×10^6 HeLa-HLA-A2 cells were trypsinized, resuspended in 2% bovine serum albumin in PBS, and sorted for GFP-positive cells by using a MoFlo cell sorter (Dako UK). Cells were then washed with PBS and incubated in prelabel medium (DMEM lacking methionine and cysteine plus 2 mM L-glutamine) for 60 min at 37°C. The cells were pulsed for 9 min with 0.5 mCi/ml ³⁵S-ProMix (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and chase media were added (RPMI 1640 medium plus 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 3 mM methionine, and 60 μM cysteine) for the indicated period. The cells were washed with PBS and lysed with 1% Triton X-100 in Tris-buffered saline (TBS), pH 7.4, containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSEF), and then they were pre-cleared for 1 h with a control antibody (mouse IgG; Dako, UK) plus protein A-Sepharose (GE Healthcare). Immunoprecipitations were performed overnight with BB7.2 and fresh protein A-Sepharose. Samples were washed six times with TBS, 0.1% Triton X-100, followed by a final wash in TBS only. Samples were eluted in 2.5% SDS, 50 mM Tris, pH 8, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and then quantified using a PhosphorImager with ImageQuant software (GE Healthcare).

³²P Labeling

For each condition, 7×10^6 HeLa-HLA-A2 cells were washed with TBS, pH 7.4, preincubated for 30 min in RPMI 1640 phosphate-free medium plus 2 mM L-glutamine, and labeled with 0.5 mCi of [³²P]orthophosphate (PerkinElmer Life and Analytical Sciences, Boston, MA) for 3 h at 37°C. Cells were then washed three times with TBS plus AEBSEF and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich). Cells were lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 5 mM MgCl₂, 50 mM Tris, pH 7.3, AEBSEF, and phosphatase inhibitor cocktail 1 and 2), and HLA-A2 was immunoprecipitated using BB7.2 as described above, with the exception that samples were washed with Nonidet P-40 wash buffer (0.1% Nonidet P-40, 5 mM MgCl₂, 50 mM Tris, pH 7.3, AEBSEF, and phosphatase inhibitor cocktails 1 and 2) after immunoprecipitation. The precipitated samples were then split into two, and one fraction was digested with endoglycosidase H (endo H) according to the manufacturer's protocol (New England Biolabs, Ipswich, MA). Samples were separated by SDS-PAGE and transferred onto nitrocellulose before quantification using a PhosphorImager with ImageQuant software. Immunoblotting was subsequently carried out with HCA2 as described above to control for equal sample loading.

Coprecipitation Assay

Four hours before harvest, cells were washed with PBS and incubated with 25 mM NH₄Cl. The cells were then washed again with PBS and trypsinized, and flow cytometry was used to assess transfection efficiency. Samples were adjusted to equal transfection efficiencies and treated with 2 mM dimethyl 3,3'-dithiobispropionimidate-2 HCl (Pierce Chemical, Rockford, IL) in 0.2 mM

triethanolamine, pH 8, for 2 h at 4°C, followed by 50 mM glycine for 30 min to stop the cross-linking reaction. Cells were lysed with PBS, 0.3% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), 0.1% SDS, and AEBSEF. One percent of the lysates (normalized for total protein) was set aside for input controls, and the remainder was precleared overnight with protein A/G-agarose (Calbiochem). Immunoprecipitation was carried out for 2–3 h by using BB7.2 and protein A/G-agarose. Samples were washed six times with TBS, 0.3% CHAPS, 0.1% SDS, and eluted with 2.5% SDS, 50 mM Tris, pH 8, followed by incubation with 150 mM dithiothreitol at 37°C for 30 min. Input controls were mock eluted with 150 mM dithiothreitol at 37°C for 30 min. Samples were analyzed by Western blotting as described above.

Electron Microscopy (EM)

For immunogold labeling of cryosections, control and AP-2-depleted HeLa-HLA-A2 cells, transfected with either Nef_{STOP} or Nef_{WT} plasmids, were sorted for GFP-positive cells as described above. Fluorescence-activated cell sorting (FACS)-sorted cells in suspension were fixed by adding an equal volume of freshly prepared 4% paraformaldehyde/0.4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After 10 min of fixation at room temperature, cells were centrifuged at $1200 \times g$, and the pellet was gently resuspended in 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and postfixed for 2 h at room temperature (Kleijmeer *et al.*, 1996). Cells were embedded in 10% gelatin at 37°C, cooled on ice, trimmed, and infused with 1.7 M sucrose/15% polyvinyl pyrrolidone overnight at 4°C. Ultrathin frozen sections were cut in a cryochamber attachment using a diamond knife mounted on a Reichert Ultracut S ultramicrotome (Leica, Milton Keynes, United Kingdom), collected with 2% methyl cellulose and 2.3 M sucrose in a 1:1 ratio, and mounted on Formvar-carbon-coated EM grids. Thawed 50- to 60-nm cryosections were labeled with HCA2, then incubated with rabbit anti-mouse antibody (Dako UK) followed by 10-nm protein A gold (Utrecht University, Utrecht, The Netherlands) (Slot and Geuze, 1983). The sections were observed in a Phillips CM 100 transmission electron microscope (Philips Electron Optics, Cambridge, United Kingdom) at an operating voltage of 80 kV.

Quantitative analysis of the intracellular distribution of HLA-A2 was determined by applying standard stereology methods (Weibel, 1979; Griffiths, 1993). The HCA2 antibody was first tested in serial dilutions on the parental cell line and HeLa-HLA-A2 cells to minimize nonspecific labeling. Grids with sections from each test sample, labeled with HCA2 at 1:75, were scrambled to allow for unbiased analysis. Grids were first examined at low magnification to find a grid square containing sections. At higher magnification (21,000 $\times$), photos were taken within randomly chosen cells. More than 100 pictures were captured of each test sample from two grids and two independent labeling experiments. The presence of gold particles associated with the plasma membrane, the nucleus, the cytoplasm, the Golgi region, lysosomes, electron-lucent vacuoles and associated tubulovesicular membranes, multivesicular bodies (MVBs) (both internal and limiting membranes), and MVB-associated tubulovesicular membranes were scored and expressed as a percentage of the total number of gold particles counted for each condition. More than 1200 gold particles were counted for each test sample, and a total of 6607 gold particles were analyzed. Golgi/TGN membranes were defined as clearly visible Golgi stacks plus tubular and vesicular membranes within a distance of 200 nm of the stack. Vacuoles were defined as possessing vacuolar morphology with an electron-lucent lumen ranging from 200 to 700 nm. MVBs were defined as typical round-membrane structures containing many tightly packed internal vesicles, whereas lysosomes were defined by their content of multilamellar ring-like structures. Tubular and vesicular membranes found within a distance of 500 nm of the electron-lucent vacuoles or MVBs were distinguished from tubulovesicular membranes found elsewhere in the cytoplasm.

Quantification of the percentage of area occupied by MVBs and electron-lucent vacuoles, their mean size, and number/100 μm² was determined as described by Weibel (1979) and Griffiths (1993). The same photos used for gold particle analysis were overlaid with a 1.5-cm grid lattice to score points within the cytoplasm, the nucleus, MVBs, and the electron-lucent vacuoles. More than 6800 points were analyzed for each test sample, and a total of 32,272 points were analyzed.

RESULTS

Assay for MHC Class I Down-Regulation

The system that we used is shown diagrammatically in Figure 1a. Because Nef is more effective at down-regulating some MHC-I isotypes than others (Le Gall *et al.*, 1998; Collins and Baltimore, 1999), for host cells we used a HeLa cell line that stably expresses HLA-A2, which has been shown to be particularly sensitive to Nef. siRNA knockdowns were started on day 1 and repeated on day 3. Transient transfections were carried out on day 4, by using an IRES plasmid encoding Nef and GFP as a bicistronic message so that we

Figure 1. Assay for Nef-induced down-regulation of MHC-I. (a) Schematic diagram of the system we used. (b and c) HLA-A2-expressing HeLa cells were transfected with either the Nef_{STOP} or the Nef_{WT} plasmid, labeled with anti-Nef, and examined by fluorescence microscopy for Nef (red) and GFP (green) labeling. Bar, 20 μm. (d) Cells similar to the cells in b and c were analyzed by flow cytometry. Nef and GFP expression have a linear relationship in cells transfected with the Nef_{WT} plasmid. (e) Cells were transfected with the indicated plasmids, labeled for surface HLA-A2, and analyzed by flow cytometry. Down-regulation of surface HLA-A2 by Nef_{LL164-165AA} is not markedly different from down-regulation by Nef_{WT}, but Nef^{EEEE62-65AAAA} has impaired activity. (f) Bar graph of results pooled from at least three independent experiments similar to that shown in e.

could identify the Nef-expressing cells (Schindler *et al.*, 2003). As a control, we used the same plasmid in which a premature stop codon had been inserted into the Nef sequence at position 2 (Nef_{STOP}). Mutations were also engineered into both the acidic cluster motif (Nef^{EEEE62-65AAAA}) and the dileucine motif (Nef^{LL164-165AA}). The cells were shifted to 26°C on day 5, because Nef-mediated MHC-I down-regulation in HeLa cells has been shown to be enhanced at this temperature (Kasper *et al.*, 2005; Supplemental Figure S1). Assays were carried out on day 6.

Figure 1, b and c, shows that cells transfected with the control plasmid (Nef_{STOP}) express GFP only, whereas cells transfected with the wild-type Nef plasmid coexpress both GFP and Nef (shown in red). To determine whether GFP expression levels increase concomitantly with Nef expression levels, cells were transfected with either Nef_{WT} or Nef_{STOP}, and then they were fixed, permeabilized, labeled with anti-Nef,

and analyzed by flow cytometry. Figure 1d shows that there is a linear relationship between GFP fluorescence and Nef staining in cells expressing wild-type Nef. Thus, GFP fluorescence is a reliable indicator of Nef expression.

To quantify surface expression of HLA-A2, we transfected the cells with Nef_{STOP}, Nef_{WT}, Nef^{EEEE62-65AAAA}, and Nef^{LL164-165AA} plasmids, and then we labeled the cells after 48 h with anti-HLA-A2 at 4°C and analyzed them by flow cytometry. Figure 1e shows representative dot plots, with the GFP fluorescence on the x-axis and the surface HLA-A2 fluorescence on the y-axis. Histogram overlays are shown below the dot plots, comparing the surface HLA-A2 fluorescence in cells expressing Nef_{WT}, Nef^{EEEE62-65AAAA}, and Nef^{LL164-165AA} (solid gray) with the surface HLA-A2 fluorescence in cells expressing GFP only (i.e., Nef_{STOP}; black line) and with the background fluorescence in cells labeled with an irrelevant primary antibody (gray line). The mean

Figure 2. Western blots of siRNA-treated cells. Equal protein loadings of homogenates of cells treated with the indicated siRNA were blotted and probed with the indicated antibodies. The $\mu 1$ and PACS-2 bands are indicated with arrows; the other bands labeled with the $\mu 1$ and PACS-2 antibodies are nonspecific.

levels of surface HLA-A2 are shown in Figure 1f. Expression of wild-type Nef causes surface HLA-A2 to drop to ~40–

50% of control levels, which is typical of what others have found using cultured cell lines (Kasper and Collins, 2003). The dileucine mutant (Nef_{LL164-165AA}) is not significantly different from wild-type Nef, whereas the acidic cluster mutant (Nef_{EEEE62-65AAAA}) shows a marked reduction in its ability to modulate surface expression of HLA-A2. Interestingly, however, the acidic cluster mutant is still partially active, especially at very high concentrations. This is consistent with a report by Williams *et al.* (2005), who showed that mutating the four glutamic acid residues to glutamines impaired the ability of Nef to reduce surface expression of HLA-A2 in a human T cell line but did not abolish it. Together, the results confirm that in our system as in other systems, Nef down-regulates HLA-A2 in a concentration-dependent manner and that the acidic cluster contributes to the down-regulation, but the dileucine is dispensable.

siRNA Knockdowns

Having established a system for quantifying Nef-induced MHC-I down-regulation, we used siRNAs to knock down various proteins. Figure 2 shows representative Western blots of cells treated with a range of siRNAs. In every case, the signal from the target protein is reduced to almost undetectable levels after knockdown.

We first investigated the involvement of AP-1, by knocking down either the γ subunit or the $\mu 1$ subunit. Because AP-1 acts in a clathrin-dependent manner, we also looked at cells that had been depleted of CHC. The top and middle panels in Figure 3a show representative dot plots from cells

Figure 3. Role of AP-1 and clathrin in down-regulation of HLA-A2 by Nef. GFP expression and surface HLA-A2 were analyzed by flow cytometry (a), and results from at least three independent experiments are plotted as a bar graph with the standard deviations shown as error bars (b). Although there was some variability in GFP (and therefore Nef) expression from one experiment to another, which accounts for the size of the error bars, the data show that knocking down either the γ or the $\mu 1$ subunit of AP-1, or CHC, inhibits Nef-induced down-regulation of HLA-A2.

Figure 4. Role of PACS-1 and PACS-2 in down-regulation of HLA-A2. GFP expression and surface HLA-A2 were analyzed by flow cytometry (a), and results from at least three independent experiments are plotted as a bar graph (b). Knocking down PACS-1 or PACS-2, either alone or in combination, has no effect on Nef-induced down-regulation of HLA-A2.

transfected with the Nef^{STOP} and Nef^{WT} plasmids, respectively. The characteristic downward slope of the Nef-expressing cells is much reduced in all three knockdowns, and the histogram overlays (Figure 3a, bottom) show much less separation between Nef-expressing (Nef^{WT}) cells and control (Nef^{STOP}) cells. Each experiment was repeated at least three times, and the mean levels of surface HLA-A2 from all the experiments are shown in Figure 3b. Together, these data show that knocking down either AP-1 γ , AP-1 μ 1, or clathrin strongly inhibits Nef-induced down-regulation of HLA-A2 in HeLa cells.

PACS-1

If Nef binds to AP-1 indirectly via PACS-1, as has been suggested previously (Piguet *et al.*, 2000), then knocking down PACS-1 should have a similar effect to knocking down AP-1 or clathrin. However, when we depleted PACS-1, we saw no effect on Nef activity (Figure 4). This result was confirmed using four additional siRNAs directed against PACS-1, including one siRNA previously reported by Crump *et al.* (2003) to be effective for knockdowns. All of the siRNAs were able to deplete PACS-1 when analyzed by Western blotting, but no effect on down-regulation of HLA-A2 was observed (unpublished data). PACS-1 has a homologue called PACS-2, which is 54% identical in sequence. Because of the possibility that PACS-1 and PACS-2 might be functionally redundant, we also tried knocking down PACS-2, either alone or in combination with PACS-1. Again, we saw no effect on Nef activity (Figure 4), even

though Western blotting showed efficient depletion of both proteins (Figure 2 and Supplemental Figure S4). Thus, neither PACS-1 nor PACS-2 seems to be required for the down-regulation of HLA-A2 by Nef.

The results of the PACS-1 depletion experiments prompted us to revisit the role of PACS-1 in the sorting of other acidic cluster proteins. We have previously shown that knocking down PACS-1 does not affect the steady state distribution of a chimera consisting of the CD8 extracellular and transmembrane domains followed by the cytoplasmic tail of carboxypeptidase D (CPD), an acidic cluster protein that normally resides in the TGN (Harasaki *et al.*, 2005). However, CPD may have more than one sorting signal in its cytoplasmic tail (Eng *et al.*, 1999). Similarly, furin, the best characterized of the acidic cluster proteins, has at least two sorting signals: the acidic cluster and YKGL, a typical YXX Φ motif that acts as an internalization and intracellular sorting signal by binding to AP complexes (Voorhees *et al.*, 1995). To examine the role of the acidic cluster motif without any contribution from the YXX Φ motif, we made use of an existing cell line expressing a CD8 chimera with the wild-type furin tail (Seaman, 2004), and we also generated two new cell lines expressing CD8-furin chimeras with mutations in the cytoplasmic tail: one in which the YKGL sorting signal was rendered nonfunctional by mutating it to AKGL (CD8-furin_{AKGL}), and another in which the acidic cluster was also mutated by changing an EEDE sequence to AAAA (CD8-furin_{AKGL-AAAA}).

Figure 5. Further investigations into the function of PACS-1 and PACS-2. (a–c) HeLa cell lines stably expressing CD8-furin chimeras either with the wild-type furin tail (a) or with mutations in the furin tail (b and c) were labeled with anti-CD8. Mutating only the YKGL motif (CD8-furin_{AKGL}) does not affect the TGN localization of the construct, but mutating both the YKGL motif and the EEDE motif (CD8-furin_{AKGL-AAAA}) causes the construct to redistribute to the cell surface. (d–g) Cells expressing the wild-type CD8-furin construct were depleted of CHC, PACS-1, PACS-2, or PACS-1 and -2. Only the clathrin knockdown affects the steady-state distribution of the construct. (h–k) Cells expressing the CD8-furin_{AKGL} construct were depleted of CHC, PACS-1, PACS-2, or PACS-1 and -2. Only the clathrin knockdown affects the steady-state distribution of the construct. Bar, 20 μ m. (l and m) Cells expressing the indicated construct were assayed for surface expression of the construct by flow cytometry. Only the clathrin knockdown significantly increases the amount of construct at the plasma membrane. (n) Western blots of CCVs from control cells and mock CCVs from cells treated with a CHC siRNA were probed with the indicated antibodies. The cell homogenate lanes contain a higher protein loading than the CCV lanes. Unlike CHC and AP-1, PACS-1 is neither enriched in CCVs nor depleted from mock CCVs. The signal from PACS-1 is only detectable in the CCV lanes after a very long exposure (right), and it is unaffected by clathrin knockdown, indicating that the small amount of PACS-1 in the preparation is a contaminant.

The steady-state distribution of the three constructs is shown Figure 5, a–c. Both CD8-furin_{WT} and CD8-furin_{AKGL} have an intracellular perinuclear distribution. In contrast, CD8-furin_{AKGL-AAAA} localizes mainly to the plasma membrane (Figure 5c), indicating that the acidic cluster is the essential sorting signal in the CD8-furin_{AKGL} construct. Next we investigated the effects of knocking down clathrin, PACS-1, and PACS-2 on the steady-state distribution of CD8-furin_{WT} and CD8-furin_{AKGL}. Clathrin knockdowns caused both constructs to take on a more peripheral distribution, with increased labeling of the plasma membrane (Figure 5, d and h). However, knocking down PACS-1 and PACS-2, either alone or in combination, had no apparent effect (Figure 5, e–g and i–k). We also carried out a more quantitative assay for increased surface expression of the constructs by labeling cells at 4°C with a CD8-specific antibody and then analyzing them by flow cytometry. Surface expression of both CD8-furin_{WT} and CD8-furin_{AKGL} increased four- to sixfold in the clathrin-depleted cells, but no significant increase was observed in the PACS-1, PACS-2, or PACS-1 + PACS-2–depleted cells (Figure 5, l and m). Together, these data indicate that PACS-1 and PACS-2 are

dispensable not only for Nef-induced down-regulation of MHC-I but also for the sorting of cargo proteins with acidic cluster motifs.

To determine whether PACS-1 is at least part of the clathrin-associated machinery, we probed Western blots of CCV preparations from HeLa cells with PACS-1 antibodies. We have previously shown that bona fide CCV proteins are not only enriched in such preparations, they are also dramatically lost from “mock CCVs” prepared from clathrin-depleted cells (Hirst *et al.*, 2004; Borner *et al.*, 2006). Figure 5n shows that whereas the AP-1 γ subunit displays this type of behavior, PACS-1 is only found in trace amounts in CCVs prepared from control cells and is not depleted from mock CCVs. This indicates that the small amount of PACS-1 present in the control CCV preparation is a contaminant.

AP-2

Although the AP-1 and clathrin knockdown data support the hypothesis that Nef keeps MHC-I off the cell surface by exploiting an intracellular pathway, there is evidence that Nef also increases the rate of endocytosis of MHC-I (Schwartz *et al.*, 1996), and it is thought that this pathway may be particularly

Figure 6. Role of AP-2 in down-regulation of HLA-A2. GFP expression and surface HLA-A2 were analyzed by flow cytometry (a), and results from at least three independent experiments are plotted as a bar graph (b). Knocking down either the α or the $\mu 2$ subunit of AP-2 enhances Nef-induced down-regulation of HLA-A2.

important in HeLa cells (Blagoveshchenskaya *et al.*, 2002). To investigate whether AP-2 might contribute to the down-regulation of MHC-I, we depleted both the α and the $\mu 2$ subunit (Figures 2 and 6). We expected to find either a reduction in Nef activity or no effect. Surprisingly, however, we found that knocking down AP-2 actually enhances Nef activity (Figure 6 and Supplemental Figure S2). At high concentrations of Nef, surface labeling for HLA-A2 approaches the background levels observed when the cells are labeled with an irrelevant antibody (Figure 6a). The effect of knocking down AP-2 is entirely dependent upon coexpression of Nef; knockdown of AP-2 has no effect on the surface expression of HLA-A2 in cells transfected with the Nef_{STOP} plasmid (Figure 6a).

At first the AP-2 knockdown results seemed counterintuitive. AP-2 facilitates clathrin-mediated endocytosis, so why would AP-2 depletion result in less HLA-A2 at the cell surface? Somehow, normal levels of AP-2 must impede the ability of Nef to down-regulate HLA-A2 in HeLa cells. The dramatic down-regulation that we observed after depleting AP-2 is reminiscent of the down-regulation that others have seen in primary T cells (Kasper and Collins, 2003), and we speculated that T cells might naturally express much less AP-2 than HeLa cells. However, when we probed Western

blots of HeLa cells and T cells with antibodies against AP-1, AP-2, and AP-3 subunits, we saw similar expression levels of AP-1 and AP-2 in the two cell types and differences only in the expression level of AP-3 (Figure 7a).

How might depleting AP-2 potentiate the ability of Nef to down-regulate MHC-I? We considered a number of possibilities, and some of our results are shown in Figure 7, b–f. One possibility was that depleting AP-2 might somehow lead to increased Nef expression and/or stability. We tested this hypothesis in two ways. First, we performed Western blots on control and AP-2–depleted cells that had been transfected with either the Nef_{STOP} or the Nef_{WT} plasmid and then sorted for GFP-expressing cells. Labeling the blots with anti-Nef showed that AP-2 depletion did not change the intensity of the Nef signal (Figure 7b). Second, we carried out flow cytometry on permeabilized cells that had been labeled with anti-Nef. Again, we saw no differences in Nef expression relative to GFP expression in the AP-2–depleted cells compared with controls (Figure 7c). Thus, knocking down AP-2 does not lead to changes in the total amount of Nef per cell.

Another possibility was that AP-2 depletion might alter the phosphorylation state of MHC-I. MHC-I can be phosphorylated *in vivo* by a cyclic AMP-dependent protein kinase (Guild and Strominger, 1984), and there is evidence that Nef only interacts with hypophosphorylated MHC-I (Kasper *et al.*, 2005). If AP-2 were involved in the trafficking of the kinase, then knocking down AP-2 might lead to an increase in hypophosphorylated HLA-A2. To test this hypothesis, we labeled cells with ³²P and immunoprecipitated cell extracts with anti-HLA-A2. Figure 7d shows that similar amounts of phosphorylated HLA-A2 come down in control and AP-2–depleted cells.

A third possibility was that AP-2 might be acting as a sink for Nef binding. If Nef is able to interact with AP-2 as well as AP-1, then depleting AP-2 might increase the amount of Nef available to bind to AP-1. Roeth *et al.* (2004) have shown that AP-1 can be immunoprecipitated with antibodies against HLA-A2 in Nef-expressing T cells, and they suggested that Nef provides a link between HLA-A2 and AP-1. We were able to repeat this result using HeLa cells (Figure 7e). To find out whether Nef also causes other AP complexes to come down with HLA-A2, we probed blots of the same samples with antibodies against the AP-2 α and AP-3 $\mu 3$ subunits. Figure 7e shows that the interaction seems to be specific for AP-1. We also carried out immunoprecipitation experiments using AP-2–depleted cells, to determine whether knocking down AP-2 increases the amount of AP-1 that coprecipitates with HLA-A2 in a Nef-dependent manner. However, we saw no differences in labeling intensity (Figure 7f). Thus, although Nef may well be binding *in vivo* to AP-2 (and AP-3) as well as to AP-1, our data indicate that the enhancement of Nef-induced down-regulation of HLA-A2 in AP-2–depleted cells is unlikely to be due simply to an increase in Nef availability.

Localization and Turnover of MHC Class I in Nef-expressing Cells

If AP-2 depletion does not affect the total amount of Nef in the cell, the phosphorylation state of MHC-I, or the interaction between MHC-I and AP-1, how else might it be acting? We postulated that knocking down AP-2 might have an indirect effect on the trafficking of MHC-I, by altering the membranes through which it passes. It has been reported that Nef causes an increase in the rate of degradation of HLA-A2 (Schwartz *et al.*, 1996; Roeth *et al.*, 2004), so first we tested whether there was even more degradation in cells that

Figure 7. Investigations into the AP-2 knock-down effect. (a) Gel and Western blots of HeLa-HLA-A2 cells and T cells, probed with antibodies against the AP-1 γ subunit, the AP-2 μ 2 subunit, and the AP-3 δ and μ 3 subunits. Only AP-3 is expressed at different levels in HeLa and T cells. (b) Blots of FACS-sorted cells transfected with the indicated construct were probed with antibodies against GFP and Nef. Knockdown of AP-2 does not significantly increase the expression of Nef. (c) Control (no knockdown) and AP-2-depleted cells, transfected with either Nef_{STOP} or Nef_{WT}, were analyzed for GFP and Nef expression by flow cytometry on permeabilized cells labeled with anti-Nef. Knocking down AP-2 does not change the ratio of GFP expression to Nef expression. (d) ³²P-labeled extracts of non-Nef-expressing cells were immunoprecipitated with anti-HLA-A2 and separated by SDS PAGE followed by Western blotting, either with or without prior digestion with endo H. After PhosphorImager analysis of ³²P, the blots were labeled with anti-HLA-A2 for enhanced chemiluminescence. Immunoglobulin heavy and light chains (Ig HC and LC) are also labeled by the secondary antibody. Knocking down AP-2 does not affect the phosphorylation state of HLA-A2. (e) Extracts of cells transfected with Nef_{STOP} and Nef_{WT} plasmids were treated with a cross-linker, and then they were immunoprecipitated with anti-HLA-A2. Coprecipitating proteins are indicated with arrows. Nef expression causes AP-1 to coprecipitate with HLA-A2, but AP-2 and AP-3 do not coprecipitate. (f) Cells were treated as described in e, except some were depleted of AP-2. Knocking down AP-2 does not affect the ability of AP-1 to coprecipitate with HLA-A2 in Nef-expressing cells.

had been depleted of AP-2. The cells were pulse labeled with ³⁵S and chased for up to 20 h, after which HLA-A2 was immunoprecipitated from cell extracts. Our results show that Nef expression causes a marked increase in the rate of degradation of HLA-A2 (Figure 8a) and also slows down the transport of HLA-A2 from the endoplasmic reticulum (ER) to the Golgi (Supplemental Figure S3). However, knocking down AP-2 did not have any obvious effect on HLA-A2 trafficking.

Next, we localized MHC-I in control and AP-2-depleted cells, both in the presence and in the absence of Nef (Figure 8, b–q). For technical reasons, the cells were not labeled with the HLA-A2-specific antibody that we used for flow cytometry, but with an antibody that recognizes all human MHC-I isotypes; however, there is evidence that HLA-A2 is the major MHC-I isotype expressed in these cells (Joyce, 1997). The cells were fixed with paraformaldehyde and labeled for surface MHC-I, and then they were permeabilized with Triton X-100 and labeled again with the same primary antibody followed by a different secondary antibody to visualize total MHC-I. The immunofluorescence images show a clear reduction in the amount of MHC-I at the plasma membrane in Nef-expressing cells, with a concomitant increase in the

labeling of intracellular membranes (Figure 8, f–i and n–q). However, at the light microscope level, we were unable to determine the exact identity of the intracellular membranes, or to distinguish any clear differences between AP-2-depleted and control cells. Therefore, we turned to electron microscopy to examine the ultrastructure of the HLA-A2-positive membranes.

For the EM studies, we used flow cytometry to sort for cells expressing high levels of GFP, and then we fixed the cells and labeled frozen thin sections with anti-HLA-A2 followed by colloidal gold. To avoid any bias in our morphometric analysis, the grids were scrambled before they were examined in the microscope. Representative micrographs are shown in Figure 9, a–d, and the percentage of label associated with different compartments in Figure 9e. Not surprisingly, the most striking difference between Nef-expressing and non-Nef-expressing cells is in the amount of HLA-A2 at the plasma membrane. In the control cells, the percentage of label at the plasma membrane goes down from ~70% in cells transfected with the Nef_{STOP} plasmid to ~4% in cells transfected with the Nef_{WT} plasmid, and in the AP-2-depleted cells transfected with the Nef_{WT} plasmid, <1% of the label is at the plasma membrane. The reason that

Figure 8. Stability and localization of HLA-A2. (a) Cells were pulse labeled with ^{35}S and chased for the indicated time, and then HLA-A2 was immunoprecipitated. Expression of Nef decreases the stability of HLA-A2; there is also a delay in ER-to-Golgi transport (see Supplemental Figure S3). Knocking down AP-2 does not have any obvious effect. (b–q) Control and AP-2-depleted cells were transfected with the indicated plasmid, and then they were labeled for surface and total MHC-I. Nef expression decreases surface expression of MHC-I and causes an increase in the labeling of a perinuclear compartment. Bar, 20 μm .

these differences are more dramatic than those we observe by flow cytometry is that we selected for high expression of GFP, instead of analyzing the entire population of GFP-expressing cells.

Nearly half of the label in the Nef_{WT} cells, and ~20% in the Nef_{STOP} cells, was found to be associated with the “cytoplasm,” which includes cytoplasmic membranes of unknown identity as well as cytoplasm where no membranes could be detected. Because the background labeling is extremely low (e.g., only 2–3% of the label is associated with the nucleus), and because HLA-A2 is an integral membrane protein, we suspect that nearly all of the cytoplasmic label is associated with membranes but that some of the membranes are out of the plane of section. These membranes are likely to include the ER, which is difficult to identify in frozen thin sections.

Although Nef has been reported to redirect MHC-I to the TGN (Piguet *et al.*, 2000; Blagoveshchenskaya *et al.*, 2002; Scott *et al.*, 2003), we saw very little labeling of Golgi membranes, including the TGN (Figure 9, b and e). Instead, about half of the label in both sets of Nef_{WT} cells is associated with membranes that seem to be endosomal in origin. These include electron-lucent vacuoles (Figure 9c), MVBs (Figure 9, b and d), and tubules associated with the vacuoles and

MVBs. Interestingly, we found that the relative amounts of MHC-I associated with vacuoles and MVBs are different in the two populations of Nef_{WT} cells: in the control cells, more label is associated with the vacuoles, whereas in the AP-2-depleted cells, more label is associated with the MVBs, in particular with internal vesicles. Table 1 shows that both of these compartments accumulate in Nef-expressing cells, as has been reported previously (Stumptner-Cuvelette *et al.*, 2003). We found that the vacuoles are more abundant in the control Nef_{WT} cells than in the AP-2-depleted Nef_{WT} cells, which partially accounts for the increase in vacuole labeling in the control Nef_{WT} cells. In contrast, the MVBs are similarly abundant in both populations of cells, so the increased labeling of MVBs in the AP-2-depleted cells represents a real shift in the steady state distribution of MHC-I. The internal vesicles of MVBs are thought to be a “point of no return,” from which proteins cannot recycle to the plasma membrane but are destined to be degraded in lysosomes (Gruenberg and Stenmark, 2004). Thus, the accumulation of MHC-I on these vesicles in the AP-2-depleted Nef_{WT} cells, as well as the slight increase in lysosomal labeling, helps to explain why there is less MHC-I at the plasma membrane.

Figure 9. Immunogold labeling of HLA-A2. (a–d) Representative electron micrographs of control and AP-2–depleted cells, transfected with Nef_{STOP} and Nef_{WT} plasmids, and sorted for high expression of GFP. Thawed cryosections of cells were labeled with anti-HLA-A2 followed by 10-nm protein A-gold. HLA-A2 is mainly on the plasma membrane in cells transfected with Nef_{STOP} (a). In cells transfected with Nef_{WT} (b–d), much of the label is associated with vacuoles and MVBs, although not with the TGN. Bar, 200 nm. (e) Quantification of the gold labeling. Nef expression causes a decrease in HLA-A2 at the plasma membrane and a concomitant increase in intracellular HLA-A2. Knocking down AP-2 causes a shift in HLA-A2 distribution from electron-lucent vacuoles to MVBs. Gold particles associated with unidentifiable membranes and other cytoplasmic structures are categorized as cytoplasm.

Table 1. Morphometric analysis of MVBs and vacuoles in micrographs of control and AP-2-depleted cells, transfected with either Nef_{STOP} or Nef_{WT}, and sorted for high expression of GFP

	Control	AP-2 kd	Control	AP-2 kd
	Nef _{STOP}	Nef _{STOP}	Nef _{WT}	Nef _{WT}
Mean size of MVB (μm^2)	0.084	0.080	0.078	0.094
Mean size of vacuoles (μm^2)	0.052	0.060	0.081	0.084
% of area occupied by MVBs	0.900	0.855	1.49	1.80
% of area occupied by vacuoles	0.137	0.125	3.42	0.78
No. of MVBs/100 μm^2	10.3	10.8	19.9	18.7
No. of vacuoles/100 μm^2	1.80	1.00	42.4	9.27

More than 100 random frames were taken from two different experiments. Numbers were determined by applying standard stereology methods. There is an increase in the number of both MVBs and vacuoles in Nef-expressing cells, but fewer vacuoles in the Nef-expressing, AP-2-depleted cells than in the Nef-expressing control cells.

DISCUSSION

The mechanisms that viruses use to down-regulate MHC-I are as varied as the viruses themselves, and include blocking the transcription of MHC-I genes, preventing newly synthesized MHC-I from leaving the ER, and ubiquitinating MHC-I at the plasma membrane so that it is endocytosed and degraded in lysosomes (Ploegh, 1998; Hewitt *et al.*, 2002; Loureiro and Ploegh, 2006). HIV-1 is one of the most intensively studied of all viruses, and a role for HIV-1 Nef in the down-regulation of MHC-I was first demonstrated >10 years ago (Schwartz *et al.*, 1996). However, there is still some uncertainty about the mechanism involved: whether endocytosis plays a significant role, where the MHC-I actually goes when it is not on the plasma membrane, and which of the host cell proteins contribute to the process. In the present study, we have used HLA-A2-expressing HeLa cells as a model system to investigate some of these issues. By knocking down various proteins with siRNA, we show that AP-1 and clathrin are both needed for down-regulation, that PACS-1 is dispensable, and that AP-2 actually impedes down-regulation.

Although HeLa cells are a useful model system, they are not the normal targets of HIV-1, and there has been some question about their relevance to viral pathogenesis (Kasper and Collins, 2003). In particular, Nef-induced down-regulation of MHC-I in HeLa cells is much less dramatic than in primary T cells (Kasper and Collins, 2003), and endocytosis has been reported to play a more important role (Blagoveshchenskaya *et al.*, 2002). Shifting HeLa cells to 26°C improves the efficiency of MHC-I down-regulation (Kasper *et al.*, 2005; Supplemental Figure S1), and this is thought to be because at lower temperatures, MHC-I traffics more slowly through the secretory pathway and thus it is more available to be diverted away from the plasma membrane (Kasper *et al.*, 2005). Consistent with previous studies (Kasper and Collins, 2003), we saw a slight increase in the rate of endocytosis of HLA-A2 in Nef-expressing cells (unpublished data); nevertheless, uptake was extremely slow compared with proteins that are internalized by clathrin-mediated endocytosis (Motley *et al.*, 2003). Most importantly, knocking down AP-1 strongly inhibits the ability of Nef to down-regulate HLA-A2, similar to the effect seen by Roeth *et al.* (2004) when they knocked down AP-1 in T cells (also see Kasper *et al.*, 2005). Thus, the same cellular machinery is used in both types of cells, indicating that insights gained from studies on HeLa cells should be widely applicable to other cell types, including T cells.

How does Nef interact with AP-1? Three regions on Nef have been implicated in MHC-I down-regulation: the acidic cluster, a proline-rich domain, and an amphipathic α -helix

near the N terminus containing a critical methionine residue (Roeth and Collins, 2006). In cells expressing a chimera consisting of Nef fused to the cytoplasmic tail of HLA-A2, AP-1 was shown to coimmunoprecipitate with the chimera, and deleting the α -helix or mutating the methionine prevented AP-1 from coimmunoprecipitating, suggesting that this part of the molecule may interact either directly or indirectly with AP-1 (Roeth *et al.*, 2004). Mutating the amphipathic α -helix has also been shown to cause the most complete block in Nef activity. However, many studies, including our own, demonstrate that the acidic cluster is also needed for efficient down-regulation of MHC-I (Greenberg *et al.*, 1998b; Piguat *et al.*, 2000; Williams *et al.*, 2005).

There are several reports claiming that the acidic cluster keeps MHC-I off the cell surface by binding to PACS-1, but for the most part, these studies have relied on overexpression of PACS-1 dominant-negative constructs (Blagoveshchenskaya *et al.*, 2002; Crump *et al.*, 2001; Scott *et al.*, 2003), and there are several examples in the literature of such constructs producing indirect effects. For example, a truncated version of the AP-2 binding partner Eps15 has been shown to be a potent inhibitor of clathrin-mediated endocytosis (Benmerah *et al.*, 1998). However, the much more modest effects of Eps15 knock-down (Huang *et al.*, 2004), and even of a complete knockout in *Caenorhabditis elegans* (Salcini *et al.*, 2001), suggest that the dominant-negative construct may work by titrating out key binding partners, such as AP-2. Similarly, overexpressing PACS-1 mutants may indirectly inhibit Nef activity by affecting other proteins. Antisense RNA directed against PACS-1 has also been reported to inhibit the down-regulation of MHC-I by Nef (Piguat *et al.*, 2000). However, the extent of PACS-1 knockdown with the antisense RNA was less than we find with siRNAs, and off-target effects are more likely with full-length antisense RNA than with short, carefully chosen duplexes (Jackson *et al.*, 2003). The present study is the first investigation into the role of PACS-1 in MHC-I down-regulation by using siRNA-induced silencing, and we saw no effect when we efficiently depleted PACS-1 by using a number of different siRNAs, nor when we depleted PACS-1 and PACS-2 together.

We also found that knocking down PACS-1 and/or PACS-2 did not affect the steady-state distribution of two CD8-furin chimeras, one of which was completely dependent upon its acidic cluster. In addition, when we probed Western blots of CCV preparations from HeLa cells with antibodies against PACS-1, we found that it was neither enriched in control CCVs nor depleted from mock CCVs prepared from clathrin-depleted cells, indicating that it is

not part of the clathrin machinery. Thus, at present the function of PACS-1 is unclear, but it may play some sort of a regulatory role relating to the phosphorylation state of acidic cluster proteins. It is also not clear what the adaptor(s) for acidic cluster motifs might be, if not PACS-1, but candidates include the AP complexes, especially because acidic clusters can function as internalization signals as well as signals for intracellular membrane traffic (Voorhees *et al.*, 1995).

Probably our most surprising result was the dramatic increase in HLA-A2 down-regulation when we depleted AP-2. We tested the role of AP-2 initially because we thought that knocking it down might inhibit Nef activity. MHC-I is normally internalized relatively slowly by a clathrin-independent pathway (Caplan *et al.*, 2002), but at least one virus, Kaposi's sarcoma virus, drives MHC-I into the clathrin-dependent pathway by ubiquitinating its cytoplasmic tail (Hewitt *et al.*, 2002). However, knocking down AP-2 had the opposite effect to the one we were expecting, and greatly enhanced the ability of Nef to down-regulate HLA-A2. This effect is not restricted to HLA-A2; we found that AP-2 knock-downs also enhanced the more modest Nef-induced down-regulation of other MHC-I isotypes endogenously expressed by the parental HeLa cell line (Supplemental Figure S2).

Why does knocking down AP-2 potentiate Nef activity? We were able to rule out a number of possibilities, and in fact the only clear difference we saw between control and AP-2-depleted cells was in the steady-state distribution of HLA-A2. This difference was not obvious by light microscopy, but by electron microscopy, we found that although HLA-A2 was localized to endosomes in both populations of cells, there was preferential localization to different endosomal subcompartments. Interestingly, almost none of the HLA-A2 was associated with the TGN, in spite of reports claiming that this is where MHC-I is localized in Nef-expressing cells (Piguet *et al.*, 2000; Blagoveshchenskaya *et al.*, 2002; Scott *et al.*, 2003). However, all of the previous studies have relied on fluorescence microscopy, and it can be difficult to distinguish the TGN from other perinuclear membranes at this level of resolution. The present study is the first to localize MHC-I in Nef-expressing cells by electron microscopy, and our observations clearly indicate that Nef directs MHC-I from the TGN to endosomes, rather than the other way around.

Our current working hypothesis is that knocking down AP-2 affects the protein composition of endosomes and that this in turn influences the fate of MHC-I, which is rerouted to endosomes in Nef-expressing cells. Several studies have shown that membrane proteins whose steady-state distribution is mainly endosomal and/or lysosomal, including the transferrin receptor, lysosomal-associated membrane proteins (LAMPs)-1 and -2, CD63, and the invariant chain of MHC class II, redistribute to the plasma membrane in AP-2-depleted cells (Dugast *et al.*, 2005; Janvier and Bonifacio, 2005; McCormick *et al.*, 2005). It has also been reported that knocking down AP-2 changes the dynamics of early endosomes, causing them to mature more rapidly into late endosomes (Lakadamyali *et al.*, 2006). These changes are consistent with our own observations. Nef expression induces the formation of electron-lucent vacuoles and MVBs, both of which are thought to be endosomal (Stumptner-Cuvelette *et al.*, 2003). However, there are fewer vacuoles relative to MVBs in AP-2-depleted cells. The vacuoles are likely to be an early population of endosomes because they do not contain any internal membranes, whereas the MVBs are filled with the internal vesicles characteristic of later endosomes (Gruenberg and Stenmark, 2004). Knocking down AP-2 al-

ters the steady state distribution of HLA-A2 in Nef-expressing cells, causing a shift from vacuoles to MVBs. Together, these findings suggest that the HLA-A2 may be targeted initially to the vacuoles, which then mature into MVBs. The acceleration of this maturation process in AP-2-depleted cells would help to keep the HLA-A2 off the cell surface, because it would limit the amount of time spent in an early endosomal compartment, from which it could be transported to the plasma membrane, and sequester it inside a late endosomal compartment, from which it would go on to be degraded in lysosomes.

Is MHC-I also localized to endosomes in Nef-expressing T cells, and if so, are they early or late? So far, this question has only been addressed by light microscopy, but Roeth *et al.* (2004) showed partial overlap of MHC-I with LAMP-1, a marker for late endosomes and lysosomes, and they also demonstrated an increase in the rate of lysosomal degradation. Thus, it seems likely that in T-cells, Nef also targets MHC-I to a late endosomal compartment as a prerequisite for degradation in lysosomes, which helps to explain why HeLa cells become more T cell-like in their response to Nef when AP-2 is depleted. In the future, it will be important to confirm and extend our findings on HeLa cells using T cells, and in particular to localize HLA-A2 in Nef-expressing T cells at the electron microscope level. Other important questions include how the HLA-A2 is sorted to the internal vesicles of MVBs (i.e., whether it is by ubiquitination and interaction with ESCRT complexes, and if so, which E3 ubiquitin ligases are involved), and precisely why knocking down AP-2 stimulates this pathway. Because AP-2 knock-downs change the steady-state distribution of so many proteins normally residing in endosomes and lysosomes, establishing which of these proteins might contribute to the enhancement of Nef activity will not be easy. However, double knockdowns of AP-2 and clathrin show that the clathrin knockdown phenotype is the dominant phenotype, indicating that the enhanced Nef activity in AP-2-depleted cells is still clathrin dependent (Supplemental Figures S4 and S5). Interestingly, preliminary observations indicate that knocking down AP-3 also enhances Nef-induced down-regulation of HLA-A2 (Supplemental Figure S5), and because T cells naturally express relatively low levels of AP-3, this provides a possible clue as to why Nef exerts a stronger effect on T cells than on HeLa cells.

Although we embarked upon this study to try to understand the mechanism of action of Nef, our findings also shed light on the mechanism of action of AP complexes. The endosomal localization of HLA-A2 in Nef-expressing cells supports a role for AP-1 in forward rather than retrograde trafficking between the TGN and endosomes. The lack of any effect on Nef-induced down-regulation of HLA-A2 when we knock down PACS-1 means that the role of PACS-1 as an adaptor for the AP-1 pathway needs to be reevaluated. The potentiation of Nef by the depletion of AP-2 (and possibly AP-3), and the inhibition of Nef by the AP-1 knockdown, suggests that different types of adaptors may have fundamentally different functions, rather than all of them contributing in a similar manner to lysosomal delivery (Pelham, 2004). The change in the steady-state distribution of HLA-A2 in the AP-2-depleted, Nef-expressing cells and the enhancement of HLA-A2 down-regulation in these cells highlight the importance of the AP-2 complex in endosomal dynamics. Thus, like many viral proteins, Nef is proving to be a valuable tool for investigating the membrane trafficking machinery of the host cell.

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