HIV Nef-mediated Major Histocompatibility Complex Class I Down-Modulation Is Independent of Arf6 Activity

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HIV Nef has a number of important biological effects, including the down-modulation of several immunological important molecules (CD4, major histocompatibility complex [MHC] class I). Down-modulation of CD4 seems to be via clathrin-dependent endocytosis, whereas down-modulation of MHC class I remains unexplained. Several mutant proteins, including mutations in the small GTPase Arf6, have been used to probe membrane traffic pathways. One such mutant has recently been used to propose that Nef acts through Arf6 to activate the endocytosis of MHC class I. Here, we show that MHC class I down-modulation is unaffected by other Arf6 mutants that provide more specific perturbations in the GDP-GTP cycling of Arf6. Inhibition of phosphatidylinositol-3-phosphate kinase, an upstream activator of Arf6, also had no effect on the internalization step, but its activity is required to direct MHC class I to the *trans*-Golgi network. We conclude that the apparent Arf6 dependency of Nef-mediated MHC class I down-modulation is due to nonspecific perturbations in membrane traffic.

INTRODUCTION

One strategy used by HIV virus to evade immune recognition of infected cells is to down-modulate the expression of MHC class I (MHC-I) at the plasma membrane by the expression of the viral protein Nef (Schwartz *et al.*, 1996; Collins *et al.*, 1998; Cohen *et al.*, 1999). Nef also induces the down-modulation of other membrane proteins such as CD4 and CD28 (Greenberg *et al.*, 1998a; Swigut *et al.*, 2000, 2001). Nef has three recognized interaction motifs involved in membrane traffic, one is a dileucine-based sequence that interacts with clathrin adaptors (Bresnahan *et al.*, 1998; Greenberg *et al.*, 1998a), a second is a PxxP-based sequence that interacts with SH3 domains (Saksela *et al.*, 1995; Grzesiek *et al.*, 1996; Lee *et al.*, 1996; Arold *et al.*, 1997; Fackler *et al.*, 1999), and a third is a EEEE-based sequence that interacts with the phosphofurin acidic cluster sorting 1 protein PACS-1 (Piguet *et al.*, 2000; Crump *et al.*, 2001). The first of these is unnecessary for MHC-I down-regulation, but both the SH3 motif and the PACS-1 interaction motif are required. CD4 down-modulation is relatively well understood; it involves the association of both Nef and CD4 with the clathrin adaptor AP-2 and the subsequent incorporation of the entire complex into clathrin-coated vesicles (Bresnahan *et al.*, 1998; Greenberg *et al.*, 1998a,b). Down-modulation of MHC-I, in contrast, does not require the interaction of Nef with AP-2 (Greenberg *et al.*, 1998a; Mangasarian *et al.*, 1999). Blockage of the clathrin endocytic pathway by overexpres-

sion of a dynamin mutant prevents Nef-mediated CD4 but not MHC-I down-modulation (Le Gall *et al.*, 2000), even though MHC-I can be found in endocytic clathrin-coated vesicles in the absence of Nef (Machy *et al.*, 1987).

The activity of the small GTPase Arf6 may be involved in Nef-dependent MHC-I internalization (Blagoveshchenskaya *et al.*, 2002). This idea initially arose from the fact that MHC-I, in the absence of Nef, is found in a vesiculo-tubular network involved in a form of clathrin-independent endocytic traffic between the plasma membrane and endosomal compartments (Radhakrishna and Donaldson, 1997; Brown *et al.*, 2001; Caplan *et al.*, 2002; Naslavsky *et al.*, 2003). The integrity of this pathway requires the function of Arf6 (Radhakrishna and Donaldson, 1997; Brown *et al.*, 2001; Caplan *et al.*, 2002; Naslavsky *et al.*, 2003). Recently, it was shown that overexpression of the constitutively active Arf6Q67L, which lacks GTPase activity, blocks Nef-mediated MHC-I down-modulation (Blagoveshchenskaya *et al.*, 2002). This observation led to the suggestion that the Nef-mediated traffic of MHC-I also involves the Arf6 vesiculo-tubular network. Further observations by Blagoveshchenskaya *et al.* (2002) led them to propose that targeting of Nef to the endosome/*trans*-Golgi network (TGN) compartment activates phosphatidylinositol-3-kinase (PI-3-kinase), which activates the guanine nucleotide exchange factor (GEF) ARNO, which in turn activates Arf6 at the plasma membrane. These events culminate in increased MHC-I internalization.

The Arf6 mutant used in this study, Arf6Q67L, has a specific defect in GTPase activity and therefore is trapped in the GTP form. As a result, GTP-activating factors (GAPs) specific for Arf6 are trapped in complex with the mutant Arf and are unavailable to endogenous Arf6, which therefore is logged in its active state. If the model suggested by

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Blagoveshchenskaya *et al.* (2002) is correct, other mutations affecting nucleotide cycling in Arf6 should modulate the effects of Nef-induced PI-3-kinase activation as well.

We therefore set out to test whether other Arf6 mutants have the predicted effect on MHC-I down-modulation. We confirmed that the constitutively active Arf6Q67L blocks MHC-I down-modulation. Surprisingly, the dominant negative Arf6T27N, which is defective in GTP loading and therefore remains complexed with GTP exchange factors, had no effect on MHC-I down-modulation. Furthermore, expression of the “fast cycling” Arf6T157A, which does not require GEF to exchange GDP for GTP, did not enhance or substitute for the effects of Nef on MHC-I internalization (Santy, 2002). These and other results suggest that the effects of Nef are unlinked to the GDP-GTP cycling of Arf6. The effect of Arf6Q67L on Nef function may be a consequence of global perturbations in the organization of the traffic system used by MHC-I rather than a specific interaction between Arf6 and Nef. Moreover, we found that the inhibition of PI3-kinase had no effect on the Nef-mediated uptake of MHC-I from the cell surface, although it does prevent the traffic of internalized MHC-I from endosomes to the TGN, in accordance with previous results (Swann *et al.*, 2001) showing that the activity of PI-3-kinase is required for retention of MHC-I in the Golgi compartment of cells expressing Nef.

MATERIALS AND METHODS

Reagents

Jurkat T-cells expressing high levels of human CD4 (a kind gift from Dr. Dan R. Littman, New York University, New York, New York) were grown in RPMI 1640 supplemented with 10% fetal bovine serum along with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml). U373mg astrocytoma cells (generously provided by Drs. Domenico Tortorella and Hidde Ploegh, Harvard Medical School, Boston, MA) and human embryonic kidney 293T cells (a kind gift from Dr. Rick van Etten, Harvard Medical School) were grown in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 mg/ml), and nonessential amino acids (0.1 mM). A purified mouse monoclonal antibody (W6/32, hereby referred to anti-MHC-I), which recognizes assembled MHC-I heavy chain- β 2 microglobulin complexes, harvested from hybridoma cells (a kind gift from Dr. Hidde Ploegh, Harvard Medical School), was fluorescently labeled with Alexa Fluor 594 fluorophores (A594-anti-MHC-I) following instructions of the manufacturer (Molecular Probes, Eugene, OR). A purified monoclonal anti- β 1-integrin antibody was kindly provided by Drs. Junichi Takagi and Timothy A. Springer (Harvard Medical School). Monoclonal hemagglutinin (HA)-antibody 12CA5 was harvested from hybridoma cells. Plasmids containing HA-tagged Arf6 (wild-type, T27N and Q67L) were kindly provided by Dr. Julie Donaldson (National Institutes of Health) and Dr. Michel Franco (Institut de Pharmacologie Moléculaire, CNRS, Valbonne, France). Plasmid containing Eps15(A95-295) fused to enhanced green fluorescent protein (EGFP) was kindly provided by Dr. Jennifer Lippincott-Schwartz (National Institutes of Health). The EHD1 cDNA (a kind gift from Dr. Mia Horowitz, Tel Aviv University, Tel Aviv, Israel) was fused with EGFP in the pEGFP-C1 vector (BD Biosciences, Clontech, Palo Alto, CA). The monomeric red fluorescent protein (Campbell *et al.*, 2002), generously provided by Dr. Roger Y. Tsien (University of California, San Diego, San Diego, CA), was fused with NefAAAA in the MIGR1 vector. Wortmannin (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) according to manufacturer's recommendations. FuGENE 6 (Roche Diagnostics, Indianapolis, IN) was used for plasmid transfections.

Mutagenesis, Retroviral Vector Constructs, Virus Preparation, and Transductions

All mutations in Nef (strain NL4-3) and Arf6 cDNAs were introduced using the QuikChange protocol (Stratagene, La Jolla, CA), and subsequently confirmed by DNA sequencing. The following mutants were generated: a form of Nef in which the methionine at position 20 was changed to alanine (NefM20A), a form of Nef in which the proline-rich P₇₂xxP₇₅ (x is any amino acid) motif was changed to A₇₂xxA₇₅ (NefAxxA), a mutant in which the acidic stretch (EEEE₆₂₋₆₅) was substituted with a quadruple alanine stretch (NefAAAA), a “fast-cycling” mutant of Arf6, Arf6T157A (Santy, 2002), and a variant of the constitutively active Arf6Q67L, referred to as Arf6N48I;Q67L, which is incapable of activating the downstream effector phospholipase D (Vitale *et al.*, 2002). The bicistronic retroviral vector MIGR1 (Pear *et al.*, 1998)

was modified to contain either a single cDNA encoding Nef fused to EGFP or two separate open reading frames, i.e., Arf6-HA and Nef-EGFP, placed upstream and downstream of an internal ribosomal entry sequence (Figure 1A). Flow cytometry and fluorescence imaging confirmed levels of Arf6-HA and Nef-EGFP. Recombinant virus was produced by simultaneous transient three-plasmid transfection of the 293T tumor cell line with the MIGR1-based constructs, a pMD.MLV vector containing murine stem cell virus Gag and Pol-genes, and the pMD.G vector containing the vesicular stomatitis virus envelope gene (Pear *et al.*, 1993; Soneoka *et al.*, 1995). Retroviral-containing supernatants were collected 36–40 h after transfection and used immediately for transduction of Jurkat T-cells (twofold dilution) or U373mg astrocytoma cells (fivefold dilution) in the presence of 4 μ g/ μ l polybrene (Sigma-Aldrich).

Flow Cytometric Analysis of MHC-I, CD4, and EGFP Expression

Flow cytometric analysis (FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, NJ) for the surface expression of CD4 or MHC-I and the total cellular content of the appropriate fusion proteins containing EGFP were performed in Jurkat T-cells (25,000 per sample). Aliquots of 25,000 cells were washed in 1% bovine serum albumin/phosphate-buffered saline and stained for 30 min on ice with diluted mouse monoclonal anti-CD4 antibody conjugated to phycoerythrin (BD PharMingen, San Diego, CA) and mouse monoclonal anti-HLA-A,B,C antibody conjugated to allophycocyanin (BD PharMingen). Excess antibody was removed by 5 washes with 1% BSA/phosphate-buffered saline (PBS) and then fixed for 15 min on ice with 3% paraformaldehyde in PBS. The rate of MHC-I internalization was determined at least three times by following the uptake of the mouse monoclonal Alexa594-anti-MHC-I as described previously (Swigut *et al.*, 2000). The role of PI-3-kinase on MHC-I internalization was determined in Jurkat T-cells pre-incubated for 1 h at 37°C with wortmannin (1 or 5 μ M) or DMSO (control), followed by its presence during the antibody-internalization assay.

Optical Microscopy

Fluorescent images were acquired with a fully motorized and computer-driven inverted wide-field epifluorescence/confocal microscope (Axiovert 200M; Carl Zeiss, Thornwood, NY) equipped with a spinning-wheel confocal head (PerkinElmer Life Sciences, Foster City, CA). Image acquisitions, three-dimensional restoration and analysis were done with the aid of SlideBook (Intelligent Imaging Innovations, Denver, CO) as described previously (Legesse-Miller *et al.*, 2003).

The astrocytoma cells were seeded at ~40–50% confluence on 18- or 25-mm coverslips 24 h before transfection or transduction and imaging experiments were performed typically 48–72 h after. The uptake of cell surface MHC-I molecules was followed at 37°C by monitoring the location of internalized A594-anti-MHC-I antibodies diluted in HMESB added to live cells. In the case of Jurkat T-cells, experiments were done with the cells in suspension, which were later sedimented on poly-lysine-coated coverslips by centrifugation at 900 $\times$ g for 5 min. Cells were then washed with PBS⁺⁺, fixed with 3% paraformaldehyde for 10 min at room temperature, and imaged. The steady-state distribution of MHC-I was determined after fixation, by incubation of the cells for 2 h at room temperature with A594-anti-MHC-I antibodies diluted in HMESB with 0.2% saponin.

RESULTS

Validation of the Expression System

Because most methods of transient protein expression are not very efficient, particularly when using Jurkat T-cells, we implemented a retroviral based bicistronic vector MIGR1 (Figure 1A) for protein expression that routinely allows transduction efficiencies of 95–100% in Jurkat T-cells and U373mg astrocytoma cells (Figure 1B). We first verified that expression of Nef (strain NL4-3) yielded the expected effects on MHC-I and CD4 down-modulation. As shown in Figure 1B, expression of the chimeric Nef protein (Nef fused at its carboxy terminus to EGFP) for 2 d in Jurkat T-cells induced substantial surface down-modulation of MHC-I and CD4. As demonstrated earlier, this effect directly correlates with the expression levels of Nef alone or Nef fused to EGFP (Greenberg *et al.*, 1998b; Swigut *et al.*, 2000; Liu *et al.*, 2001). The down-modulation of MHC-I involves a fourfold increase in its rate of internalization (Figure 1C), well within the range obtained by others using transiently transfected Jurkat or HeLa cells (Swigut *et al.*, 2000; Blagoveshchenskaya *et al.*, 2002). As expected from previous work (Greenberg *et al.*, 1998b; Mangasarian *et al.*, 1999; Akari *et al.*, 2000; Piguet

Figure 1. Down-modulation of MHC-I molecules induced by Nef in Jurkat T-cells and U373mg astrocytoma cells. (A) Jurkat T-cells were transduced with retroviral constructs to express either EGFP, or the chimeric proteins Nef, NefM20A, NefAxxA, or NefAAAA fused to EGFP for 48 h. The extent of MHC-I and CD4 molecules at the cell surface and the expression level of EGFP were determined by flow cytometry and are plotted on a logarithmic scale. For each experiment, 25,000 cells were analyzed. (B) MHC-I rates of internalization in Jurkat T-cells in the absence of Nef or expressing wild-type and mutant Nef proteins, determined as the percent fraction of cell surface MHC-I molecules internalized as a function of time. (C) Uptake of MHC-I in U373mg astrocytoma cells expressing wild-type or mutants forms of Nef. 72 h after transduction, cells were incubated at 37°C with Alexa594-anti-MHC-I for 4 h, and with Alexa647-transferrin during the last 30 min. Each image corresponds to single confocal sections of randomly selected fields of cells. Bar, 20 μ m.

et al., 2000; Blagoveshchenskaya *et al.*, 2002), expression of NefM20A, NefAxxA, or NefAAAA induced a robust down-modulation in the surface expression of CD4, but not of MHC-I (Figure 1B). Similarly, direct visualization of surface MHC-I and its uptake (monitored by following the internalization of Alexa594-anti-MHC-I, a fluorescently labeled antibody specific for the ectodomain of MHC-I) in astrocytoma cells expressing Nef-EGFP showed the expected decrease in surface expression of MHC-I and the accumulation of internalized MHC-I in the perinuclear region (Figure 1D) and the decrease in the intracellular pool of MHC-I (our unpublished data). As expected, expression of NefM20A, NefAxxA, or NefAAAA in astrocytoma cells had no influence in the traffic of MHC-I. Together, these results validate the

use of the viral vector as an efficient system for protein expression in Jurkat and astrocytoma cells.

Nef and the Intracellular Traffic of MHC-I

To follow the internalization pathways of MHC-I in more detail, we incubated the astrocytoma cells with the MHC-I antibody in the absence or presence of Nef. In the absence of Nef, a fraction of the anti-MHC-I antibodies labeled the cell periphery, including colocalization with clathrin and its endocytic adaptor AP-2, whereas another fraction was internalized into an early endosomal compartment. The early endosome compartment was identified by being labeled with the early endosomal marker EEA1 (Figure 2A) and by

Figure 2. Nef and the intracellular traffic of MHC-I. (A) In the absence of Nef, a fraction of MHC-I colocalizes with clathrin, its endocytic adaptor AP-2 and the early endosome marker EEA1. Confocal images of U373mg astrocytoma cells transiently expressing yellow fluorescent protein (YFP)-tagged clathrin light-chain A for 20 h were incubated with Alexa594 anti-MHC-I antibodies for 25 min at 37°C and then cells were washed, fixed and visualized for clathrin light chain, or stained with an antibody specific for AP-2 (second row) or EEA1 (third row). Arrows indicate examples of colocalization. (B) In the presence of Nef, the fraction of MHC-I internalized for down-modulation does not involve the clathrin-endocytic pathway. Images of U373mg astrocytoma cells transiently expressing EGFP-Eps15- Δ 95-295 alone (top row), or together with Nef-CFP (1:5 DNA ratio) (bottom row) for 72 h were incubated with Alexa594-anti-MHC-I for 4 h at 37°C, and with Alexa647-hTf during the last 30 min. Bar, 20 μ m.

containing fluorescent transferrin taken up by clathrin-dependent receptor-mediated endocytosis (our unpublished data). A similar distribution of MHC-I was observed in cells expressing the Nef mutants NefM20A, NefAxxA, or NefAAAA, which do not induce MHC-I down-modulation (our unpublished data). These observations confirm previous suggestions from electron microscopy (Machy *et al.*, 1987) that in the absence of Nef MHC-I can be internalized via the clathrin-dependent pathway.

In the presence of Nef, however, internalized MHC-I trafficked not only to transferrin-containing endosomes but also reached the TGN (see below, Figure 5C). It is known that the surface down-modulation of MHC-I does not require a

clathrin-dependent pathway (Greenberg *et al.*, 1998a; Le Gall *et al.*, 2000). Confirming this, overexpression of the dominant negative Eps15 Δ 95-295 form of Eps15, a specific inhibitor for the clathrin endocytic pathway (Benmerah *et al.*, 1999), had no effect on the Nef-induced traffic of MHC-I to the TGN (Figure 2B). In the absence of Nef, a small fraction of cells (1–5%) displayed a vesiculo-tubular network containing internalized MHC-I antibody (Figure 3). The frequency of this phenotype was similar in astrocytoma cells expressing wild-type Nef, NefM20A, or NefAxxA. Unexpectedly, the expression of NefAAAA results in a substantial increase (10–20%) in the number of cells displaying the vesiculo-tubular network. Similar expansion of the tubular network was observed with Jurkat T-cells (Figure 2C). The tubules observed in the astrocytoma cells expressing NefAAAA were long (20–60 μ m) and thin (<0.5 μ m), and occurred as bundles, some linking the perinuclear compartment with the plasma membrane, some emanating from the plasma membrane, and others from the endosomal compartment (Figure 3A). Most tubes aligned with microtubules (Figure 3B), and their existence was abolished by incubation of the cells with 10 μ M Nocodazole for 1 h (not shown). Membrane-bound tubes have previously been observed in studies of the internalization of membrane proteins, including MHC-I and β 1-integrins, and are known to provide a clathrin-independent mode of traffic that is sensitive to the nucleotide status of Arf6 (Radhakrishna and Donaldson, 1997; Brown *et al.*, 2001). The tubes we observed contained all the predicted membrane proteins, including EHD1, an important regulator of Arf6-regulated membrane recycling (Caplan *et al.*, 2002; Galperin *et al.*, 2002), but not transferrin (Figure 3A).

Arf6 Is Not Directly Involved on the Nef-mediated Down-Modulation of MHC-I

It has been proposed that the Arf6-regulated membrane-trafficking pathway is responsible for the increased down-modulation of MHC-I in response to Nef (Blagoveshchenskaya *et al.*, 2002). This model rests in part on the observation that Nef-dependent down-modulation of MHC-I, but not CD4, is blocked by expression of Arf6Q67L, a form of Arf6 that remains in the active state by poorly hydrolyzing GTP (D'Souza-Schorey *et al.*, 1995), which disrupts the vesiculo-tubular network (Radhakrishna and Donaldson, 1997). We therefore examined the effects of Arf6T27N, a form of Arf6 that is unable to exchange GDP for GTP and that, by scavenging the available GEF, acts as a dominant negative mutant for the traffic through the vesiculo-tubular network (Radhakrishna and Donaldson, 1997). As shown in Figure 4, A and C, expression of Arf6T27N in Jurkat and astrocytoma cells at levels similar to those of Arf6Q67L had no detectable effect on MHC-I down-modulation in response to Nef. It also had no effect on the rate of internalization of MHC-I in Jurkat T-cells in the presence of Nef (Figure 4B). Next, we tested the effect of Arf6T157A, a fast-cycling form of Arf6 that undergoes spontaneous GDP-GTP exchange (Santy, 2002). If the model is correct, expression of Arf6T157A should mimic the effect of Nef on MHC-I internalization. We saw no effect of Arf6T157A on MHC-I traffic, either in the presence or absence of Nef (Figure 4; our unpublished data). Finally, we expressed Nef together with Arf6N48I;Q67L, a form of Arf6 that remains in the active state, but fails to activate at least one of its known downstream effectors, phospholipase D (Vitale *et al.*, 2002). As illustrated in Figure 4A, expression in Jurkat T-cells of Arf6N48I;Q67L did not prevent the Nef-mediated surface down-modulation of MHC-I, suggesting that the interference of Arf6Q67L on MHC-I traffic is not related to the GTP/GDP cycle of Arf6.

Figure 3. Expansion of a vesiculo-tubular network by expression of NefAAAA. Expression of NefAAAA in U373mg astrocytoma cells results in the appearance of a tubular network labeled with internalized MHC-I or β 1-integrin, and stained with the regulator EHD1 but not with internalized transferrin. (A) Confocal images of U373mg astrocytoma cells transduced for 3 d with NefAAAA-EGFP (top three rows) or for 5 d with NefAAAA-RFP in cells transfected

Activity of PI-3-Kinase Is Required for the Nef-mediated MHC-I Retention in TGN

Although it seems that the GTP cycle of Arf6 is not important in regulating Nef-dependent MHC-I internalization, it is still possible that PI-3-kinase may be involved. Wortmannin is an inhibitor of PI-3-kinase that, at the concentrations used here (1–5 μ M), is not known to affect the activity of any other kinases. Incubation with wortmannin for 2.5 h had no effect on the surface and intracellular levels of MHC-I in Nef-expressing astrocytoma cells (Figure 5A). We also found that wortmannin had no effect on the rate of internalization of MHC-I in Nef-expressing Jurkat cells (Figure 5B). This treatment did, however, clearly reduce the amount of MHC-I retained in the TGN in astrocytoma cells (Figure 5C) or Jurkat T-cells (our unpublished data) even though the amount of MHC-I internalized into the endosomal compartment did not vary. These results indicate that PI-3-kinase has an important role in directing internalized MHC-I from endosomes to the TGN but does not influence the early stages of MHC-I traffic mediated by Nef.

DISCUSSION

Nef seems to modulate at least two independent endocytic pathways, a clathrin-dependent pathway, which is important for the down-modulation of CD4 (Greenberg *et al.*, 1997, 1998a; Bresnahan *et al.*, 1998) and a clathrin-independent pathway, which is important for the down-modulation of MHC-I (Le Gall *et al.*, 2000; Swann *et al.*, 2001). Different motifs on Nef are involved in these two functions; the clathrin-adaptor-binding motif is important for CD4 down-modulation (Greenberg *et al.*, 1997, 1998a; Bresnahan *et al.*, 1998; Mangasarian *et al.*, 1999), whereas the SH3- and PACS-1-binding motifs and the methionine at position 20 are involved in MHC-I down-modulation (Greenberg *et al.*, 1998b; Akari *et al.*, 2000; Piguet *et al.*, 2000; Crump *et al.*, 2001; Blagoveshchenskaya *et al.*, 2002). Arf6 modulates membrane trafficking between the plasma membrane and a nonclathrin-derived endosomal compartment (Radhakrishna and Donaldson, 1997; Brown *et al.*, 2001; Naslavsky *et al.*, 2003). The observation that Arf6Q67L prevents the Nef-mediated down-modulation of MHC-I, but not of CD4, led to the conclusion that Nef modulates MHC-I traffic by affecting Arf6 activity (Blagoveshchenskaya *et al.*, 2002). Observations that an inhibitor of PI-3-kinase LY294002 also inhibits

for 2 d with EGFP-EHD1 (bottom row). Live cells were incubated at 37°C with Alexa594-anti-MHC-I (4 h, first row), Alexa594-transferrin (30 min, second row), antibodies against β 1 integrin receptors (4 h, third row), or only medium (fourth row). The intensity of the selected regions (stippled lines) were normalized and presented in colors (first column) or in an inverted monochrome scale (last two columns). Arrows highlight the location of tubules containing MHC-I, β 1-integrin, and EHD1 molecules in the NefAAAA-enhanced tubular compartment. (B) The tubular network colocalizes with microtubules containing tubulin A (arrow heads). A three-dimensional stack of 60 consecutive epifluorescence optical sections was restored by constrained iterative deconvolution (step size 0.15 μ m, 63 $\times$, 1.4 numerical aperture objective lens), and normalized surface rendered views are shown (Volocity). Bar, 20 μ m. (C) The vesiculo-tubular network in Jurkat T-cells expressing NefAAAA. A three-dimensional stack of 67 consecutive epifluorescence optical sections (step size 0.15 μ m, 63 $\times$, 1.4 numerical aperture objective lens) was restored by constrained iterative deconvolution (Slide-Book), and normalized cross-sections x-y-z (left image) and x-y (right image) views generated with Volocity (Improvision) are shown. Bar, 10 μ m.

Figure 4. Nef-mediated MHC-I down-modulation is independent of Arf6 activity. (A) Jurkat T-cells were transduced for 48 h with wild-type Nef in combination with wild-type Arf6, Arf6Q67L, Arf6N48I;Q67L, Arf6T27N, or Arf6T157A, and analyzed for cell surface MHC-I and CD4 molecules, and expression of Nef fused to EGFP as in Figure 1A. The expression levels of Arf6 and its mutants were determined to be equivalent by Western blot analysis (our unpublished data). (B) Effect of selected Arf6 mutants in the rate of MHC-I internalization induced by Nef. The experiments were performed as described in Figure 1B. (C) Effect of selected Arf6 mutants in the uptake of MHC-I in U373mg astrocytoma cells. Spinning disk confocal sections of cells transduced to express for 72 h Nef either with wild-type Arf6 or with the mutant forms Arf6T27N, Arf6Q67L, or Arf6T157A. The cells were then incubated with Alexa594-anti-MHC-I for 4 h at 37°C, and with Alexa647-Transferrin during the last 30 min. Bar, 20 μ m.

MHC-I down-modulation allowed a further elaboration of the model to include upstream activation of PI-3-kinase by Nef as the first step in the pathway leading to Arf6 activation.

In agreement with Blagoveshchenskaya *et al.* (2002), we found that overexpression of Arf6Q67L completely blocked the down-modulation effects of Nef on MHC-I but not on CD4. When we examined other mutants, however, that should have similar or opposite effects to the Arf6Q67L mutant, we instead found no effect. The Arf6Q67L mutant

poorly hydrolyzes GTP and remains bound to GTPase-activating proteins, preventing the GTP/GDP cycle required for Arf6 function. The Arf6T27N mutant is defective in GTP loading, thus scavenging all available Arf6GEFs and the Arf6T157A mutant is able to spontaneously load GTP, bypassing the requirement for Arf6GEF. Neither of these mutants inhibited or enhanced MHC-I internalization. Similarly, there was no effect on the down-modulation of MHC-I upon expression of Arf6T27N and Nef, although expression

Figure 5. Effect of wortmannin on MHC-I traffic. (A) The steady-state distribution of MHC-I in U373mg astrocytoma cells expressing by transduction EGFP or Nef is not affected by incubation at 37°C for 2.5 h incubation with 1 μ M wortmannin, or 4 h with 20 μ M LY294002 (our unpublished data). (B) The internalization rate of MHC-I in Jurkat T-cells expressing Nef is not affected by treatment of the cells for 1.5 h with wortmannin. (C) Treatment of U373mg astrocytoma cells with wortmannin prevents the traffic of internalized MHC-I to the TGN. Seventy-two hours after transduction of U373mg astrocytoma cells with Nef, cells were treated either with 1 μ M wortmannin or with DMSO (the carrier) for 30 min at 37°C, followed by incubation with Alexa594-anti-MHC-I in the presence of wortmannin or DMSO for another 2 h. Then samples were washed, fixed, permeabilized, and stained with an antibody specific for the TGN marker TGN46. The degree of colocalization between MHC-I and the Golgi marker TGN46 was estimated from the spatial correlation of fluorescence intensities of MHC-I and TGN46 for ~30 cells per condition. Representative spinning disk confocal sections are shown. Bar, 20 μ m.

of the Arf6T27N mutant alone was shown to inhibit recycling of MHC-I in A7 melanoma cells (Blagoveshchenskaya *et al.*, 2002). Arf6 activates phospholipase D (Massenburg *et al.*, 1994; Vitale *et al.*, 2002), an enzyme used in a number of membrane-budding events (Ktistakis *et al.*, 1996; Shen *et al.*, 2001). It is particularly interesting that Arf6N48I;Q67L, a double mutant that is expected to remain in the active GTP state but fails to activate certain downstream effectors such as phospholipase D (Vitale *et al.*, 2002), does not inhibit MHC-I down-modulation. Because this double mutant should scavenge GAPs in the same way that Arf6Q67L does, it is hard to reconcile this result with the hypothesis that GTP cycling on Arf6 is required to down-modulate MHC-I. Expression of the Arf6T27N and Arf6Q67L mutants clearly interfere with Fc-mediated phagocytosis in macrophages (Zhang *et al.*, 1998) and leukocyte migration (Weber *et al.*, 2001). The results from the use of these mutants make it clear that there is a direct link between the GDP-GTP cycling of Arf6 and the biological process under investigation. In contrast, we do not believe that a similar relationship has been established between Nef and Arf6.

How then, can one rationalize the inhibitory effect of Arf6Q67L in MHC-I traffic? Arf6Q67L has two main effects: it sequesters GAPs, causing the endogenous Arf6 proteins to remain in the active form for longer; and it causes chronic activation of all the downstream effectors of Arf6. We suggest that the combined disruptions resulting from Arf6Q67L expression, GAP sequestration, and activation of down-

stream effectors causes nonspecific perturbations in membrane traffic that are not related to the GTP/GDP cycle of Arf6. We argue that the use of this mutant alone should not be thought of as sufficient evidence to identify processes that depend on Arf6 activity.

What role does PI-3-kinase play in MHC-I traffic? Blagoveshchenskaya *et al.* (2002) found that Nef stimulated the GTP loading of Arf6 and that this stimulation was blocked by addition of LY294002, a PI-3-kinase inhibitor that also blocks MHC-I down-modulation in response to Nef. The NefAxxA mutant, which lacks the SH3-binding motif, does not induce MHC-I down-modulation, but a chimeric protein in which NefAxxA is fused to the catalytic subunit of PI-3-kinase (p110) does. Together, these results seemed to support a model in which Nef recruits PI-3-kinase to the endosome/TGN, PI-3-kinase activates the GEF ARNO, and Arf6 becomes activated, resulting in increased MHC-I internalization (Blagoveshchenskaya *et al.*, 2002). Given that the Arf6 GTP/GDP cycle now seems not to be relevant to MHC-I internalization, it is possible that PI-3-kinase may be acting via some other mechanism, not relevant to Arf6. Although Blagoveshchenskaya *et al.* (2002) chimeric Nef-PI-3-kinase experiment indicates that PI-3-kinase may be sufficient to induce MHC-I entry under some circumstances, our data showing that wortmannin does not inhibit MHC-I internalization indicate that PI-3-kinase is not required for the increase of MHC-I internalization mediated by wild-type Nef. This interpretation is consistent with observations by

others showing that PI-3 kinase activity is not required for MHC-I uptake in the absence of Nef (Naslavsky *et al.*, 2003). PI-3-kinase does, however, play a role in membrane traffic involving the endosomal and Golgi compartments (Spiro *et al.*, 1996; Lauvrak *et al.*, 2002; Naslavsky *et al.*, 2003). We find that inhibition with wortmannin or with LY294002 (Swann *et al.*, 2001) prevent retention of internalized MHC-I in the TGN, a step that is required for MHC-I down-modulation (Greenberg *et al.*, 1998b; Piguet *et al.*, 2000). Intriguingly, expression of the NefAAAA mutant, which cannot interact with PACS-1 thereby preventing targeting and retention of MHC-I at the Golgi, nevertheless causes a dramatic increase in the Arf6 vesiculo-tubular network. We propose that this mutant could be useful to understand the functional relationships between Nef and Arf6. Further experiments will be required to test this possibility.

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