Molecular Biology of the Cell
Vol. 15, 323–331, January 2004

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

Jakob E. Larsen,* Ramiro H. Massol,* Thomas J. F. Nieland, and Tomas Kirchhausen†

Department of Cell Biology and The Center for Blood Research, Harvard Medical School, Boston, Massachusetts 02115

Submitted August 11, 2003; Revised September 3, 2003; Accepted September 10, 2003
Monitoring Editor: Juan Bonifacino

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 major histocompatibility complex (MHC) class 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 src homology (SH)3 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 overexpression 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 locked in its active state. If the model suggested by 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

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03–08–0578. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03–08–0578.

* These authors contributed equally to this work.
† Corresponding author. E-mail address: kirchhausen@crystal.harvard.edu.

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 PI-3-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, NY) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum along with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml). U373 mg 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% fetal bovine serum, 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-β2 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-β1-integrin antibody was kindly provided by Drs. Junichi Takagi and Timothy A. Springer (Harvard Medical School). Monoclonal HA-antibody, 12CA5, was harvested from hybridoma cells. Plasmids containing HA-tagged Arf6 (wild type, T27N, and Q67L) were kindly provided by Drs. Julie Donaldson (National Institutes of Health, Bethesda, MD), and Michel Franco (Institut dePharmacologie Moleculaire, CNRS, Valbonne, France). Plasmid containing Eps15(Δ95–295) 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 (mRFP) (Campbell *et al*., 2002), generously provided by Dr. Roger Y. Tsien (University of California, San Diego, La Jolla, 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 $P_{72}xxP_{75}$ (x is any amino acid) motif was changed to $A_{72}xxA_{75}$ (NefAxxA), a mutant in which the acidic stretch ($EEEE_{62-65}$) 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 (PLD) (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 (IRES) (Figure 1A). Flow cytometry and fluorescence imaging confirmed levels of Arf6-hemagglutinin (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 U373 mg astrocytoma cells (fivefold dilution) in the presence of 4 μg/μl 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/sample). Aliquots of 25,000 cells were washed in 1% bovine serum albumin/PBS 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 five washes with 1% bovine serum albumin/PBS and then fixed for 15min 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 preincubated for 1h at 37°C with wortmannin (1 or 5 μM) 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 (Applied Biosystems, 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 HMEMB medium 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 5min. Cells were then washed with PBS^{++} (containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$), 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 HMEMB medium 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 U373 mg 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 *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

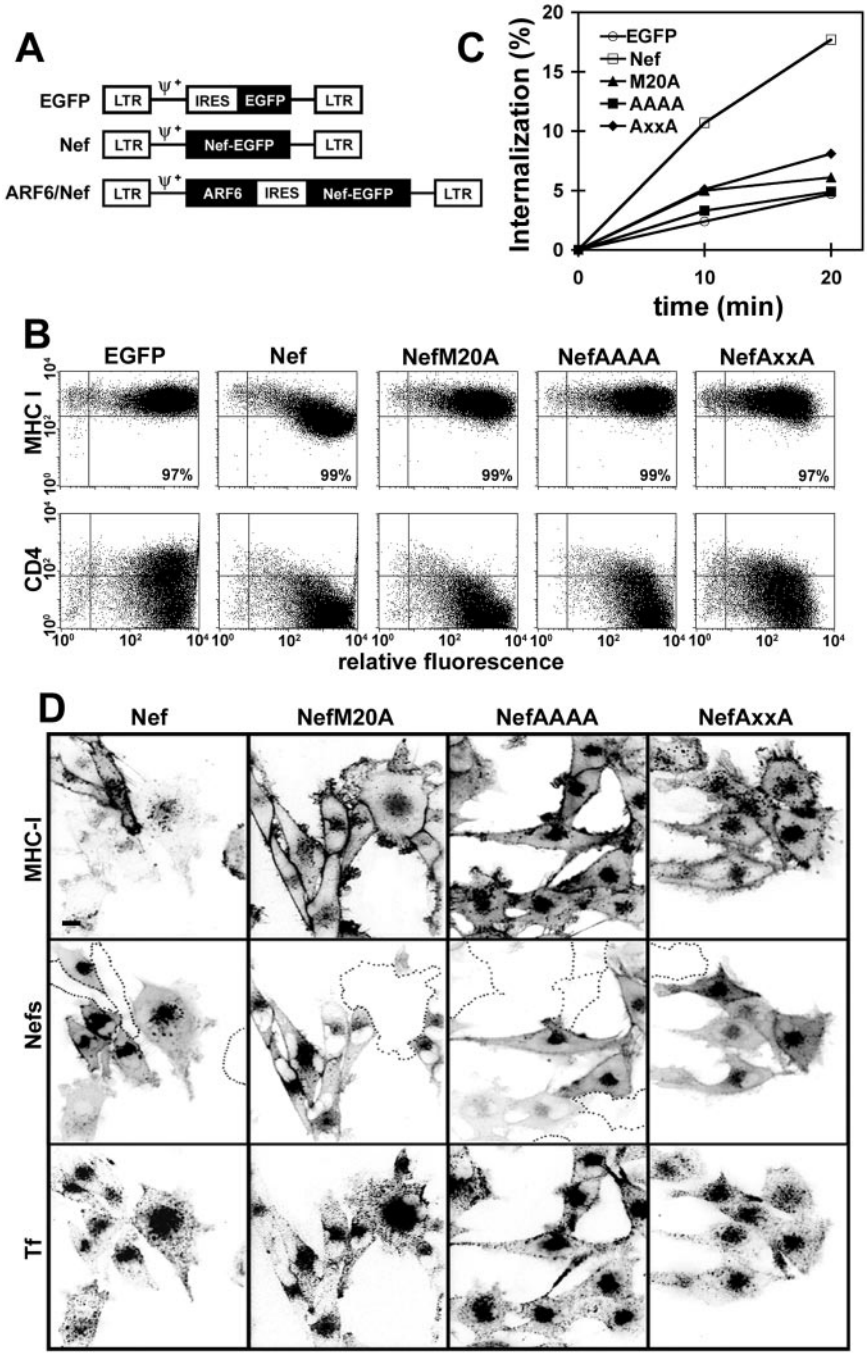

Figure 1. Down-modulation of MHC-I molecules induced by Nef in Jurkat T cells and U373 mg astrocytoma cells. (A) Jurkat T cells were transduced with retroviral constructs to express either EGFP, or the chimera proteins Nef, NefM20A, NefAxxA, or NefAAAA fused to EGFP for 48 h. (B) The extent of down-regulation 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. (C) 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. (D) Uptake of MHC-I in U373 mg astrocytoma cells expressing wild-type or mutants forms of Nef. 72h 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 μm.

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 within transferrin-containing endosomes (Figure 1D), and in the TGN (see below; Figure 5C) together with the decrease in the total 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 containing fluorescent transferrin taken up by clathrin-dependent receptor-mediated endocytosis (our unpublished data). A similar distribution of MHC-I was observed in cells

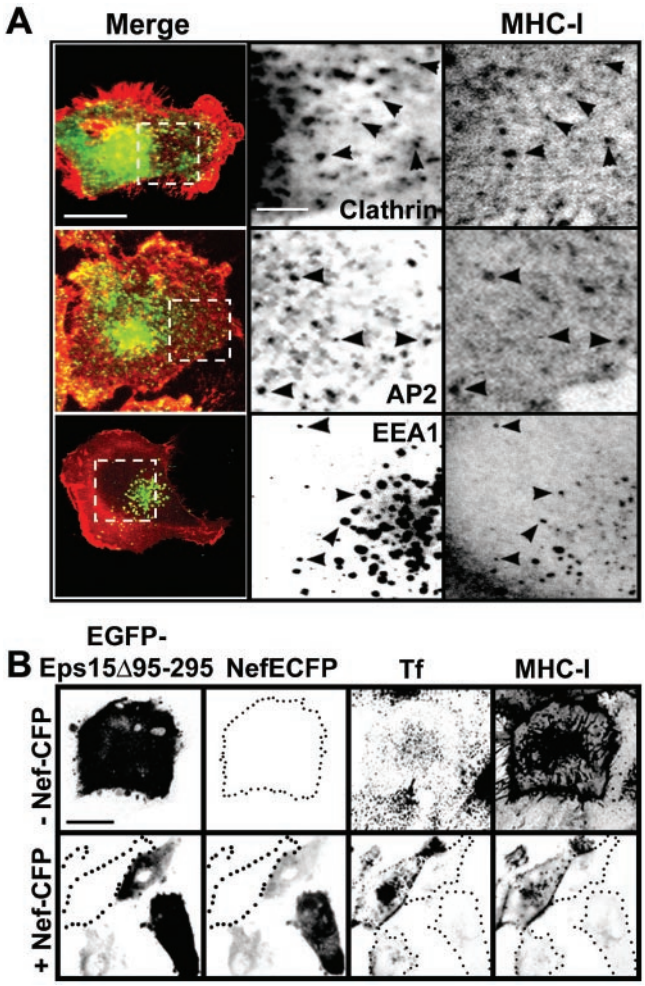

Figure 2. Nef and the intracellular traffic of MHC-I. A) In the absence of Nef, a fraction of MHC-I colocalizes with clathrin, its endocytic adaptor AP-2 and the early endosome marker EEA1. Confocal images of U373 mg astrocytoma cells transiently expressing yellow fluorescent protein-tagged clathrin 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 U373 mg astrocytoma cells transiently expressing EGFP-Eps15-Δ95-295 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 4h at 37°C, and with Alexa647-hTf during the last 30 min. The cell surface signal of MHC-I is higher in cells expressing EGFP-Eps15-Δ95-295, which interferes with internalization through the clathrin pathway. The cell contour (stippled lines) of nontransfected cells is indicated. Bar, 20 μm.

expressing the Nef mutants NefM20A, NefAxxA, or Nef AAAA, 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 Eps15Δ95-295 form of Eps15, a specific inhibitor for the clathrin endocytic pathway (Benmerah *et al*., 1999), had no effect on the Nef-induced down-modulation of MHC-I (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 3C). The tubules observed in the astrocytoma cells expressing NefAAAA were long (20–60 μm) and thin (<0.5 μm), and formed 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 μM Nocodazole for 1 h (our unpublished data). Membrane-bound tubes have previously been observed in studies of membrane proteins including MHC-I and β1-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), and tube formation was blocked by coexpression of wild-type Arf6, Arf6Q67L, or Arf6T157A.

Arf6 Is Not Directly Involved in 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 B, 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 4C). 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, it is known that Arf6N48I fails to activate at least one of its known downstream effectors, PLD, whereas it still activates PI4P5-kinase (Jones *et al*., 1999; Skippen *et al*., 2002) and interacts normally with its own GEFs and GAPs showing no impairment in GTP loading and hydrolysis (Vitale *et al*., 2002). Thus, we expressed Nef together with ArfN48I;

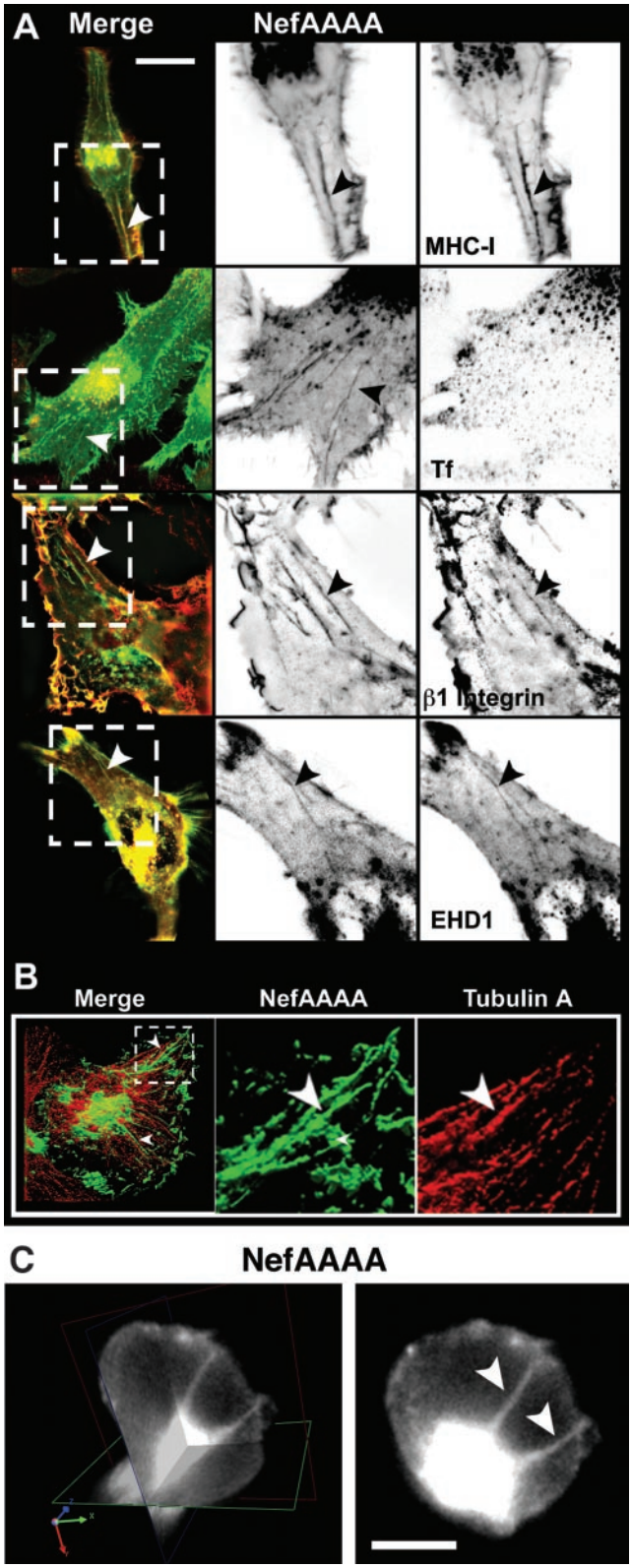

Figure 3. Expansion of a vesiculo-tubular network by expression of NefAAAA. Expression of NefAAAA in U373 mg astrocytoma cells results in the appearance of a tubular network labeled with internalized MHC-I or β1-integrin, and stained with the regulator EHD1 but not with internalized transferrin. (A) Confocal images of U373 mg astrocytoma cells transduced for 3 d with NefAAAA-EGFP (top three rows), or for 5 d with NefAAAA-RFP in cells transfected 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-transferin (30 min, second row), antibodies against β1 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, β1-integrin, and EHD1 molecules in the NefAAAA-enhanced tubular compartment. (B) Tubular network colocalizes with microtubules containing tubulin A (arrowheads). A three-dimensional stack of 60 consecutive epifluorescence optical sections was restored by constrained iterative deconvolution (step size 0.15 μm, 63×, 1.4 numerical aperture objective lens), and normalized surface rendered views are shown (Volocity). Bar, 20 μm. (C) Vesiculo-tubular network in Jurkat T cells expressing NefAAAA. A three-dimensional stack of 67 consecutive epifluorescence optical sections (step size 0.15 μm, 63×, 1.4 numerical aperture objective lens) was restored by constrained iterative deconvolution (SlideBook), and normalized cross sections x-y-z (left image) and x-y (right image) views generated with Volocity (Improvision, Lexington, MA) are shown. Bar, 10 μM.

Q67L, a form of Arf6 that should remain in the active state, but fail to activate PLD. As illustrated in Figure 4A, expression in Jurkat T cells of Arf6N48I;Q67L and Nef 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. As a control experiment, expression in astrocytoma cells of Arf6N48I;Q67L and Nef results in the appearance of the same type of small vacuoles and protrusions observed upon expression of Arf6Q67L and Nef (our unpublished data), consistent with the suggestion that Arf6N48I;Q67L is locked in the active state.

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 p85/p110 PI-3-kinase that, at the concentrations used here (0.5–5 μM), also inhibits the related VPS34 PI-3-kinase (reviewed in Siddhanta *et al*., 1998; Futter *et al*., 2001), but 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 or trafficked into 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 wortmannin is active in our experiments and 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; Bresnahan *et al*., 1998; Greenberg *et al*., 1998a) and a clathrin-independent pathway, which is important for the down-modulation of MHC-I (Le Gall *et al*., 2000; Swann *et al*., 2001).

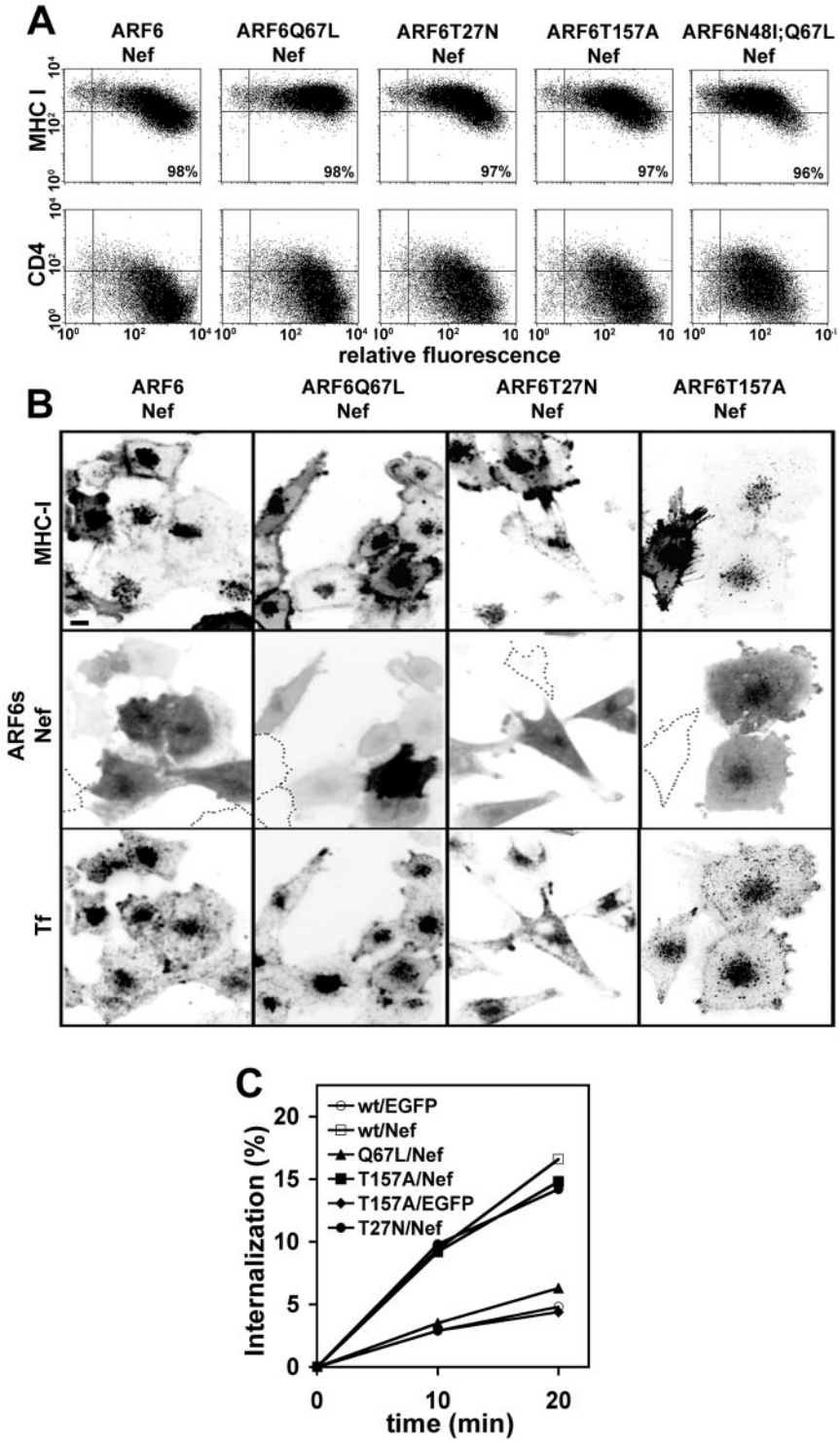

Figure 4. Nef-mediated MHC-I down-modulation is independent of Arf6 activity. (A) Jurkat T cells were transduced for 48 h with wild-type Nef in combination with wild-type Arf6, Arf6Q67L, Arf6N48I;Q67L, Arf6T27N, or Arf6T157A, and analyzed for cell surface MHC-I and CD4 molecules, and expression of Nef fused to EGFP as described 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 U373 mg 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 μm.

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 MHC-I down-modulation allowed a further elaboration of the model to include upstream activation of PI-3-kinase by

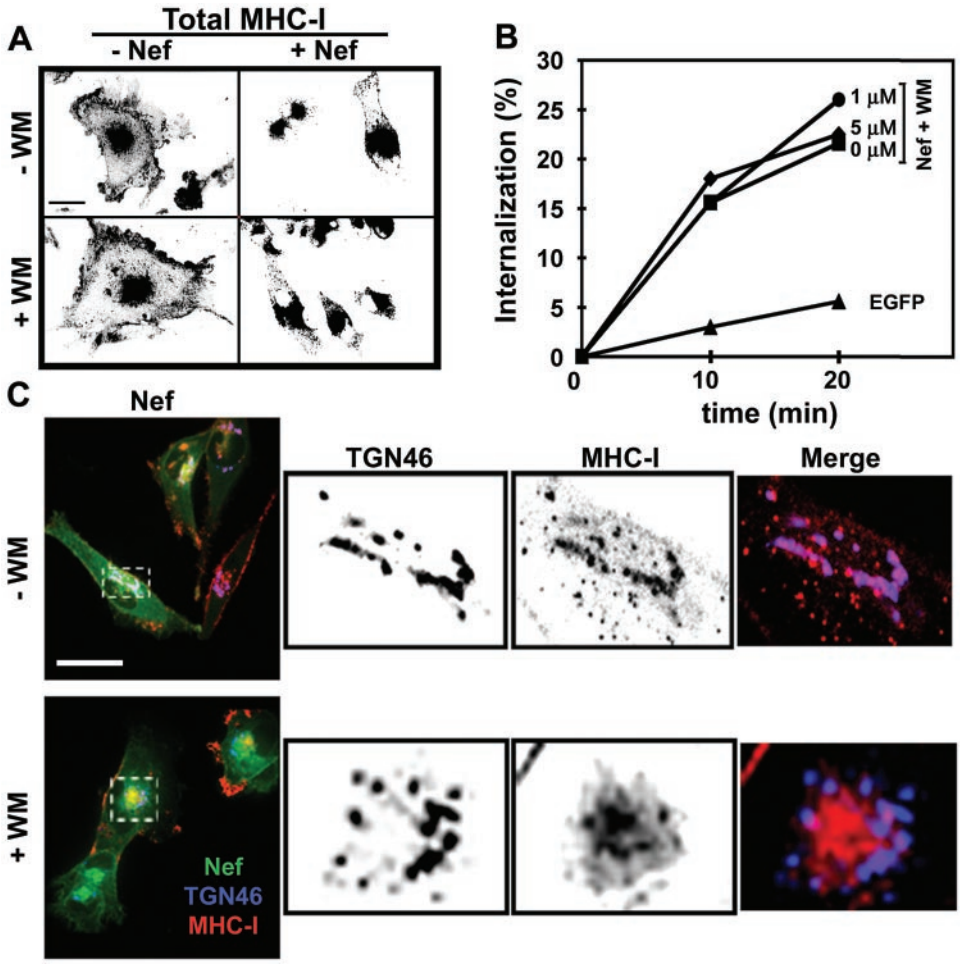

Figure 5. Effect of wortmannin on MHC-I traffic. (A) Steady-state distribution of MHC-I- in U373 mg astrocytoma cells expressing by transduction EGFP or Nef is not affected by incubation at 37°C for 2.5-h incubation with 1 μM wortmannin, or 4 h with 20 μM LY294002 (our unpublished data). (B) Internalization rate of MHC-I in Jurkat T cells expressing Nef is not affected by treatment of the cells for 1.5h with wortmannin. (C) Treatment of U373 mg astrocytoma cells with wortmannin prevents the traffic of internalized MHC-I to the TGN. Seventy-two hours after transduction of U373 mg astrocytes with Nef, cells were treated either with 1 μM (or 0.5 μM; our unpublished data) 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 μm.

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 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 downstream 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 the 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-kinases do, 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), particularly VPS34 that is involved in the regulation of membrane traffic between the Golgi and vacuoles/lysosomes (reviewed in DeCamilli *et al*., 1996; Herman *et al*., 1992; Sheperd *et al*., 1995). We find that inhibition with 0.5–1 μM wortmannin or with LY294002 (Swann *et al*., 2001) prevents 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). Because the same amount of wortmannin inhibits the enzymatic activity of p85/p110 or VPS34 (Siddhanta *et al*., 1998; Futter *et al*., 2001), however, it is not possible to use this inhibitor to distinguish the relative contribution of these kinases to the traffic of MHC-I to the TGN. 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.

ACKNOWLEDGMENTS

We thank Drs. Julie G. Donaldson, Michel Franco, Mia Horowitz, Jennifer Lippincott-Schwartz, Dan R. Littman, Hidde Ploegh, Timothy A. Springer, Junichi Takagi, Domenico Tortorella, Roger Y. Tsien, and Rick van Etten for generously providing us with DNA constructs and antibodies. We also thank Drs. Marcelo Ehrlich, Eric Macia, and Lewis Cantley for helpful discussions and Dr. Rebecca Ward for editorial assistance. Supported in part by National Institutes of Health grant (GM-60357) (to T.K.) and by the Danish Medical Research Council, the Danish AIDS Foundation, and the Leo Nielsen and Karen Margrethe Nielsen Foundation (to J.E.L.).

REFERENCES

Akari, H., Arold, S., Fukumori, T., Okazaki, T., Strebel, K., and Adachi, A. (2000). Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J. Virol. *74*, 2907–2912.

Arold, S., Franken, P., Strub, M.P., Hoh, F., Benichou, S., Benarous, R., and Dumas, C. (1997). The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling. Structure *5*, 1361–1372.

Benmerah, A., Bayrou, M., Cerf-Bensussan, N., and Dautry-Varsat, A. (1999). Inhibition of clathrin-coated pit assembly by an Eps15 mutant. J. Cell Sci. *112*, 1303–1311.

Blagoveshchenskaya, A.D., Thomas, L., Feliciangeli, S.F., Hung, C.H., and Thomas, G. (2002). HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. Cell *111*, 853–866.

Bresnahan, P.A., Yonemoto, W., Ferrell, S., Williams-Herman, D., Geleziunas, R., and Greene, W.C. (1998). A dileucine motif in HIV-1 Nef acts as an internalization signal for CD4 downregulation and binds the AP-1 clathrin adaptor. Curr. Biol. *8*, 1235–1238.

Brown,F.D.,Rozelle,A.L.,Yin,H.L.,Balla,T.,andDonaldson,J.G.(2001).Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. J. Cell Biol. *154*, 1007–1017.

Campbell, R.E., Tour, O., Palmer, A.E., Steinbach, P.A., Baird, G.S., Zacharias, D.A., and Tsien, R.Y. (2002). A monomeric red fluorescent protein. Proc. Natl. Acad. Sci. USA *99*, 7877–7882.

Caplan, S., Naslavsky, N., Hartnell, L.M., Lodge, R., Polishchuk, R.S., Donaldson, J.G., and Bonifacino, J.S. (2002). A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. EMBO J. *21*, 2557–2567.

Cohen, G.B., Gandhi, R.T., Davis, D.M., Mandelboim, O., Chen, B.K., Strominger, J.L., and Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity *10*, 661–671.

Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature *391*, 397–401.

Crump, C.M., Xiang, Y., Thomas, L., Gu, F., Austin, C., Tooze, S.A., and Thomas, G. (2001). PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic. EMBO J. *20*, 2191–2201.

De Camilli, P., Emr, S.D., McPherson, P.S., and Novick, P. (1996). Phosphoinositides as regulators in membrane traffic. Science *271*, 1533–1539.

D'Souza-Schorey, C., Li, G., Colombo, M.I., and Stahl, P.D. (1995). A regulatory role for ARF6 in receptor-mediated endocytosis. Science *267*, 1175–1178.

Fackler, O.T., Luo, W., Geyer, M., Alberts, A.S., and Peterlin, B.M. (1999). Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. Mol Cell *3*, 729–739.

Futter, C.E., Collinson, L.M., Backer, J.M., and Hopkins, C.R. (2001). Human VPS34 is required for internal vesicle formation within multivesicular endosomes. J Cell Biol *155*, 1251–1264.

Galperin, E., Benjamin, S., Rapaport, D., Rotem-Yehudar, R., Tolchinsky, S., and Horowitz, M. (2002). EHD *3*, a protein that resides in recycling tubular and vesicular membrane structures and interacts with EHD1. Traffic *3*, 575–589.

Greenberg, M., DeTulleo, L., Rapoport, I., Skowronski, J., and Kirchhausen, T. (1998a). A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for downregulation of CD4. Curr. Biol. *8*, 1239–1242.

Greenberg, M.E., Bronson, S., Lock, M., Neumann, M., Pavlakis, G.N., and Skowronski, J. (1997). Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. EMBO J. *16*, 6964–6976.

Greenberg, M.E., Iafrate, A.J., and Skowronski, J. (1998b). The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. EMBO J. *17*, 2777–2789.

Grzesiek, S., Bax, A., Clore, G.M., Gronenborn, A.M., Hu, J.S., Kaufman, J., Palmer, I., Stahl, S.J., and Wingfield, P.T. (1996). The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase. Nat. Struct. Biol. *3*, 340–345.

Herman, P.K., Stack, J.H., and Emr, S.D. (1992). An essential role for a protein and lipid kinase complex in secretory protein sorting. Trends Cell Biol *2*, 363–368.

Jones, A.T., Spiro, D.J., Kirchhausen, T., Melancon, P., and Wessling-Resnick, M. (1999). Studies on the inhibition of endosome fusion by GTPgammaS-bound ARF. J Cell Sci *112(Pt 20)*, 3477–3485.

Ktistakis, N.T., Brown, H.A., Waters, M.G., Sternweis, P.C., and Roth, M.G. (1996). Evidence that phospholipase D mediates ADP ribosylation factor-dependent formation of Golgi coated vesicles. J. Cell Biol. *134*, 295–306.

Lauvrak, S.U., Llorente, A., Iversen, T.G., and Sandvig, K. (2002). Selective regulation of the Rab9-independent transport of ricin to the Golgi apparatus by calcium. J. Cell Sci. *115*, 3449–3456.

Le Gall, S., Buseyne, F., Trocha, A., Walker, B.D., Heard, J.M., and Schwartz, O. (2000). Distinct trafficking pathways mediate Nef-induced and clathrin-dependent major histocompatibility complex class I down-regulation. J. Virol. *74*, 9256–9266.

Lee, C.H., Saksela, K., Mirza, U.A., Chait, B.T., and Kuriyan, J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell *85*, 931–942.

Legesse-Miller, A., Massol, R.H., and Kirchhausen, T. (2003). Constriction and dnm1p recruitment are distinct processes in mitochondrial fission. Mol. Biol. Cell *14*, 1953–1963.

Liu, X., Schrager, J.A., Lange, G.D., and Marsh, J.W. (2001). HIV Nef-mediated cellular phenotypes are differentially expressed as a function of intracellular Nef concentrations. J. Biol. Chem. *276*, 32763–32770.

Machy, P., Truneh, A., Gennaro, D., and Hoffstein, S. (1987). Major histocompatibility complex class I molecules internalized via coated pits in T lymphocytes. Nature *328*, 724–726.

Mangasarian, A., Piguet, V., Wang, J.K., Chen, Y.L., and Trono, D. (1999). Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. J. Virol. *73*, 1964–1973.

Massenburg, D., Han, J.S., Liyanage, M., Patton, W.A., Rhee, S.G., Moss, J., and Vaughan, M. (1994). Activation of rat brain phospholipase D by ADP-ribosylation factors 1, 5, and *6*, separation of ADP-ribosylation factor-dependent and oleate-dependent enzymes. Proc. Natl. Acad. Sci. USA *91*, 11718–11722.

Naslavsky, N., Weigert, R., and Donaldson, J.G. (2003). Convergence of non-clathrin- and clathrin-derived endosomes involves Arf6 inactivation and changes in phosphoinositides. Mol. Biol. Cell *14*, 417–431.

Pear, W.S., Miller, J.P., Xu, L., Pui, J.C., Soffer, B., Quackenbush, R.C., Pendergast, A.M., Bronson, R., Aster, J.C., Scott, M.L., and Baltimore, D. (1998). Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. Blood *92*, 3780–3792.

Pear, W.S., Nolan, G.P., Scott, M.L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA *90*, 8392–8396.

Piguet, V., Wan, L., Borel, C., Mangasarian, A., Demaurex, N., Thomas, G., and Trono, D. (2000). HIV-1 Nef protein binds to the cellular protein PACS-1 to down-regulate class I major histocompatibility complexes. Nat. Cell Biol. *2*, 163–167.

Radhakrishna, H., and Donaldson, J.G. (1997). ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. J. Cell Biol. *139*, 49–61.

Saksela, K., Cheng, G., and Baltimore, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO J. *14*, 484–491.

Santy, L.C. (2002). Characterization of a fast cycling ADP-ribosylation factor 6 mutant. J. Biol. Chem. *277*, 40185–40188.

Schwartz, O., Marechal, V., Le Gall, S., Lemonnier, F., and Heard, J.M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. *2*, 338–342.

Shen, Y., Xu, L., and Foster, D.A. (2001). Role for phospholipase D in receptor-mediated endocytosis. Mol. Cell. Biol. *21*, 595–602.

Shepherd, P.R., Soos, M.A., and Siddle, K. (1995). Inhibitors of phosphoinositide 3-kinase block exocytosis but not endocytosis of transferrin receptors in 3T3-L1 adipocytes. Biochem Biophys Res Commun *211*, 535–539.

Siddhanta, U., Mcllroy, J., Shah, A., Zhang, Y., and Backer, J.M. (1998). Distinct roles for the p110alpha and hVPS34 phosphatidylinositol 3′kinases in vesicular trafficking, regulation of the actin cytoskeleton, and mitogenesis. J Cell Biol *143*, 1647–1659.

Skippen, A., Jones, D.H., Morgan, C.P., Li, M., and Cockcroft, S. (2002). Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. J Biol Chem *277*, 5823–5831.

Soneoka, Y., Cannon, P.M., Ramsdale, E.E., Griffiths, J.C., Romano, G., Kingsman, S.M., and Kingsman, A.J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. *23*, 628–633.

Spiro, D.J., Boll, W., Kirchhausen, T., and Wessling-Resnick, M. (1996). Wortmannin alters the transferrin receptor endocytic pathway in vivo and in vitro. Mol. Biol. Cell *7*, 355–367.

Swann, S.A., Williams, M., Story, C.M., Bobbitt, K.R., Fleis, R., and Collins, K.L. (2001). HIV-1 Nef blocks transport of MHC class I molecules to the cell surface via a PI 3-kinase-dependent pathway. Virology *282*, 267–277.

Swigut, T., Iafrate, A.J., Muench, J., Kirchhoff, F., and Skowronski, J. (2000). Simian and human immunodeficiency virus Nef proteins use different surfaces to down-regulate class I major histocompatibility complex antigen expression. J. Virol. *74*, 5691–5701.

Swigut, T., Shohdy, N., and Skowronski, J. (2001). Mechanism for down-regulation of CD28 by Nef. EMBO J. *20*, 1593–1604.

Vitale, N., Chasserot-Golaz, S., Bailly, Y., Morinaga, N., Frohman, M.A., and Bader, M.F. (2002). Calcium-regulated exocytosis of dense-core vesicles requires the activation of ADP-ribosylation factor (ARF)6 by ARF nucleotide binding site opener at the plasma membrane. J. Cell Biol. *159*, 79–89.

Weber, K.S., Weber, C., Ostermann, G., Dierks, H., Nagel, W., and Kolanus, W. (2001). Cytohesin-1 is a dynamic regulator of distinct LFA-1 functions in leukocyte arrest and transmigration triggered by chemokines. Curr. Biol. *11*, 1969–1974.

Zhang, Q., Cox, D., Tseng, C.C., Donaldson, J.G., and Greenberg, S. (1998). A requirement for ARF6 in Fcgamma receptor-mediated phagocytosis in macrophages. J. Biol. Chem. *273*, 19977–19981.