The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes

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Nef, a regulatory protein of human and simian immunodeficiency viruses, downregulates cell surface expression of both class I MHC and CD4 molecules in T cells by accelerating their endocytosis. Fibroblasts were used to study alterations in the traffic of class I MHC complexes induced by Nef. We found that Nef downregulates class I MHC complexes by a novel mechanism involving the accumulation of endocytosed class I MHC in the *trans*-Golgi, where it colocalizes with the adaptor protein-1 complex (AP-1). This effect of Nef on class I MHC traffic requires the SH3 domain-binding surface and a cluster of acidic amino acid residues in Nef, both of which are also required for Nef to downregulate class I MHC surface expression and to alter signal transduction in T cells. Downregulation of class I MHC complexes from the surface of T cells also requires a tyrosine residue in the cytoplasmic domain of the class I MHC heavy chain molecule. The requirement of the same surfaces of the Nef molecule for downregulation of surface class I MHC complexes in T cells and for their accumulation in the *trans*-Golgi of fibroblasts indicates that the two effects of Nef involve similar interactions with the host cell machinery and involve a molecular mechanism regulating class I MHC traffic that is common for both of these cell types. Interestingly, the downregulation of class I MHC does not require the ability of Nef to colocalize with the adaptor protein-2 complex (AP-2). We showed previously that the ability of Nef to colocalize with AP-2 correlates with the ability of Nef to downregulate CD4 expression. Our observations indicate that Nef downregulates class I MHC and CD4 surface expression via different interactions with the protein sorting machinery, and link the sorting and signal transduction machineries in the regulation of class I MHC surface expression by Nef.

Keywords: class I MHC/HIV/Nef/SH3/*trans*-Golgi network

Introduction

Nef, a regulatory protein of primate immunodeficiency viruses (SIV and HIV), is an important determinant of AIDS pathogenesis. Viral loads remain low and AIDS rarely develops in Rhesus monkeys infected with *nef*-deleted SIV (Kestler *et al.*, 1991). Some cases of non-

progressive HIV-1 infection have also been associated with deletions in *nef* (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995). The function of Nef in AIDS pathogenesis is not known, but Nef has a number of effects on cell function that suggest how Nef might be important for viral replication and persistence *in vivo*, including the perturbation of signal transduction pathways, and the downregulation of both CD4 and class I MHC expression on the cell surface.

Nef modulates signal transduction in several cell types (Luria *et al.*, 1991; De *et al.*, 1994; Du *et al.*, 1995; Graziani *et al.*, 1996). In T cell lines Nef blocks an early event in CD3-initiated signaling and early gene expression (Luria *et al.*, 1991; Baur *et al.*, 1994; Collette *et al.*, 1995; lafrate *et al.*, 1997). Several biochemical interactions have been identified which may underlie these functions. The conserved well-structured core of the HIV-1 Nef protein mediates functional interactions with a number of proteins involved in signal transduction, including the SH3 domains of Src-family protein tyrosine kinases (Saksela *et al.*, 1995; Grzesiek *et al.*, 1996a; Lee *et al.*, 1996; Arold *et al.*, 1997; Moarefi *et al.*, 1997) and a kinase from the p21-activated kinase (PAK) family (Sawai *et al.*, 1994, 1995; Nunn and Marsh, 1996). The interactions of Nef with components of the signal transduction machinery could be important for the replication of HIV and SIV in primary cells, particularly in resting or suboptimally stimulated T cells (Miller *et al.*, 1994; Spina *et al.*, 1994; Du *et al.*, 1995; Saksela *et al.*, 1995).

Another effect of Nef is to downregulate expression of CD4 on the cell surface (Garcia and Miller, 1991; Anderson *et al.*, 1993; Benson *et al.*, 1993; Mariani and Skowronski, 1993). CD4 is a component of the T-cell receptor complex with roles in antigen-dependent signal transduction, as well as being a component of the cellular receptor for HIV and SIV (for recent review see Littman, 1996). The importance of CD4 downregulation by Nef *in vivo* is not known, but the established roles of CD4 in immunodeficiency virus infection suggest that through this effect Nef could promote viral particle release or influence other aspects related to the assembly and entry of viral particles.

Nef induces the downregulation of CD4 by accelerating its endocytosis, having no detectable effect on CD4 synthesis or transit through the exocytic pathway (Aiken *et al.*, 1994; Rhee and Marsh, 1994; Sandfridson *et al.*, 1994). Endocytosed CD4 molecules then accumulate in early endosomes and are degraded in an acidic compartment (Schwartz *et al.*, 1995). The mechanism by which Nef accelerates CD4 endocytosis likely involves the direct recruitment of CD4 to the clathrin AP-2 complex since: (i) the effect of Nef requires a dileucine motif in the membrane-proximal region of the cytoplasmic domain of CD4 (Aiken *et al.*, 1994; Salghetti *et al.*, 1995; Hua and Cullen, 1997), which is also required for the endocytosis of CD4 under other conditions (Shin *et al.*, 1991) that

occurs via clathrin-coated pits (Pelchen-Matthews *et al.*, 1993; Marsh and Pelchen-Matthews, 1996); (ii) HIV-1 Nef fusion proteins are concentrated in AP-2-containing clathrin-coated regions of the plasma membrane (Greenberg *et al.*, 1997; Mangasarian *et al.*, 1997); and (iii) in Nef-expressing cells CD4 redistributes to AP-2 clathrin coats, and this requires residues in the cytoplasmic domain of CD4 (Greenberg *et al.*, 1997). The interaction of Nef with AP-2 complexes appears to be independent from that with CD4 since they map to different surfaces within the two disordered loops in the HIV-1 Nef molecule. Mutations in the C-terminal disordered region of Nef disrupt colocalization of Nef with AP-2 (Greenberg *et al.*, 1997), suggesting that this loop links HIV-1 Nef to a component of the coat, possibly the AP-2 adaptor protein complex itself. One functional interaction with CD4 was mapped to the N-terminal disordered loop in HIV-1 Nef (Greenberg *et al.*, 1997; Iafrate *et al.*, 1997), but additional elements within the core region of the Nef molecule may also be involved (Grzesiek *et al.*, 1996b; Liu *et al.*, 1997). The recruitment of CD4 to the AP-2 complex at the plasma membrane may involve direct interactions of Nef with CD4 (Grzesiek *et al.*, 1996b; Hua and Cullen, 1997).

Another effect which has clear implications for the function of Nef *in vivo* is the recently discovered ability of Nef to downregulate cell surface expression of class I MHC molecules (Schwartz *et al.*, 1996). Surface class I MHC molecules present peptides processed from proteins synthesized within the same cell to cytotoxic T lymphocytes. This mechanism allows for the destruction of cells expressing foreign antigens and is the chief component of the acquired anti-viral immune response (reviewed by Kagi *et al.*, 1996). By interfering with class I MHC expression Nef protects infected primary cells against killing by cytotoxic T lymphocytes (Collins *et al.*, 1998), and thereby could promote the survival of HIV and SIV infected cells *in vivo* (Schwartz *et al.*, 1996).

The biogenesis of functional class I MHC complexes is relatively well understood, owing in part to the abundance of viral gene products that disrupt their assembly and transport to the cell surface (reviewed by Heemels and Ploegh, 1995; York and Rock, 1996; Fruh *et al.*, 1997; Wiertz *et al.*, 1997). Functional class I MHC complexes, containing the class I MHC heavy chain, β_2 -microglobulin and a peptide, are assembled in the ER and possibly in the *cis*-Golgi. Correctly folded heterotrimeric class I MHC complexes are released to the exocytic pathway for transport to the plasma membrane. The lifespan of functional class I MHC complexes on the plasma membrane is determined by the stability of the heterotrimeric complex and by the rate of endocytosis of the mature complexes. In T cells and macrophages the half-life of class I MHC molecules on the cell surface is relatively short compared with B-lymphocyte and fibroblast cell lines, and this probably reflects the constitutive endocytosis of class I MHC complexes via AP-2-containing clathrin-coated pits (Tse and Pernis, 1984; Machy *et al.*, 1987; Dasgupta *et al.*, 1988; Vega and Strominger, 1989; for review see York and Rock, 1996). The functional significance of this cell type-specific regulation is unclear. Internalized class I MHC complexes are either recycled to the cell surface (Abdel Motal *et al.*, 1993), or degraded.

Although little is known about how Nef downregulates class I MHC, Nef does not seem to have an effect on the synthesis and transport of class I MHC through the *cis*-Golgi. Internalized complexes were reported to accumulate in endosomal vesicles and, due to the abnormally short half-life of class I MHC in Nef-expressing cells (Schwartz *et al.*, 1996), probably degraded. In this report we investigate alterations in the normal trafficking of class I MHC induced by Nef, and the molecular interactions of Nef required to induce these alterations. We found that the ability of Nef to downregulate class I MHC expression requires an intact SH3 domain-binding surface in the Nef core. It also requires a cluster of acidic amino acid residues in Nef that is reminiscent of acidic motifs which mediate the retention of proteins in the *trans*-Golgi and are involved in the recruitment of the Golgi-associated AP-1 adaptor complexes to *trans*-Golgi membranes. Surprisingly, mutations that disrupt the ability of Nef to colocalize with AP-2 adaptor complexes at the plasma membrane do not affect the downregulation of class I MHC. In Nef-expressing fibroblasts class I MHC is retrieved from the cell surface and accumulates in the *trans*-Golgi, in vesicles containing AP-1 adaptor complex. Our observations define a novel mechanism for downregulating class I MHC expression and indicate that Nef downregulates CD4 and class I MHC cell surface expression via different sets of molecular interactions and possibly using different components of the protein sorting machinery.

Results

Nef induces the accumulation of class I MHC complexes in the Golgi

Immunofluorescence microscopy was used to study the effect of transiently expressed Nef on the distribution of class I MHC complexes. Since class I MHC internalization and flow through the endocytic pathway is rapid in T cells (Machy *et al.*, 1987), studying alterations in the class I MHC traffic induced by Nef is difficult. Therefore, we used human IMR90 fibroblasts, in which constitutive class I MHC internalization is less rapid and whose flat morphology and relatively high cytoplasm content facilitates the identification of various sorting compartments. We constructed a vector that directs expression from a bicistronic transcript of both NA7, a genetically and functionally well-characterized natural HIV-1 Nef protein (Mariani and Skowronski, 1993; Salghetti *et al.*, 1995; Greenberg *et al.*, 1997; Iafrate *et al.*, 1997), and the green fluorescent protein (GFP). This construct takes advantage of an internal ribosome entry site (IRES) to direct translation of GFP, which acts as a marker to detect Nef-expressing cells. The distribution of class I MHC complexes was revealed with the W6/32 mAb (Barnstable *et al.*, 1978). This antibody detects assembled class I heavy chains in complex with β_2 -microglobulin, but not free or denatured class I heavy chains (Jackson *et al.*, 1992). As shown in Figure 1, in the Nef-expressing GFP-positive cells the W6/32 mAb stained the perinuclear region intensely (panels 4–6). In contrast, in cells transfected with a control vector expressing only GFP, the W6/32 mAb revealed a diffuse class I MHC distribution throughout the cells and a more concentrated staining pattern in the perinuclear region of the cells (Figure 1,

Fig. 1. Effect of Nef on the distribution of class I MHC complexes in IMR90 cells. Human IMR90 fibroblasts transfected with 10 μ g of plasmids expressing the GFP marker alone (panels 1–3), or HIV-1 NA7 Nef and the GFP marker from the same bicistronic transcription unit, were fixed and class I MHC complexes were detected with W6/32 mAb, and the immune complexes were visualized by indirect immunofluorescence with polyclonal anti-mouse IgG goat antibody labeled with Texas Red (panels 3 and 6). GFP was detected by direct fluorescence (panels 1 and 4). The overlays of GFP and class I MHC images were produced using Oncor imaging software (panels 2 and 5).

panels 1–3). The decreased intensity of fluorescence throughout the periphery of Nef-expressing cells probably reflected downregulation of class I MHC expression from the cell surface by Nef, while the increased staining of the perinuclear region suggested that Nef may promote accumulation of class I MHC in the Golgi.

Class I MHC complexes colocalize with AP-1 adaptor complexes in the *trans*-Golgi in Nef-expressing cells

To confirm that class I MHC complexes accumulated in the Golgi in cells expressing Nef, we performed a series of colocalization experiments with class I MHC and proteins known to reside in the Golgi, including mannosidase II, TGN46 and the γ -subunit of AP-1 adaptor complexes. As shown in Figure 2A, the mannosidase II-specific 53FC3 mAb (Burke *et al.*, 1982) produced a perinuclear staining pattern, consistent with a previous report that mannosidase II localizes to the *medial*-Golgi (Rabouille *et al.*, 1995) (panel 3). Although both class I MHC and mannosidase II localized in the same general area of the perinuclear region, there was little colocalization of the two patterns (Figure 2A, compare panels 1, 2 and 3). Thus, the class I MHC complexes appear to accumulate mostly in mannosidase II-negative regions of the Golgi.

In contrast, the pattern of class I MHC fluorescence closely overlapped the pattern of TGN46 fluorescence (Figure 2A, panels 4–6). TGN46 is located in the *trans*-Golgi and the *trans*-Golgi network (TGN) (Hickinson *et al.*, 1997). Class I MHC also colocalized extensively with the γ -adaptin subunit of the AP-1 complex (Figure 2A, panels 7–9), which is also located in the *trans*-Golgi and in vesicles linking the *trans*-Golgi with the endocytic

pathway (reviewed by Kirchhausen *et al.*, 1997; Traub and Kornfeld, 1997). Examination of the magnified images demonstrated colocalization of the two proteins in vesicular structures (Figure 2A, compare panels 10 and 12). We observed accumulation of class I MHC in association with these structures only in Nef-expressing cells, and not in cells transfected with a control vector, even following upregulation of class I MHC expression by γ -interferon to facilitate detection of class I MHC complexes (Figure 2B). We conclude that Nef promotes accumulation of class I MHC in a γ -adaptin positive *trans*-Golgi compartment and in transport vesicles containing AP-1 adaptor complexes.

Since class I MHC complexes colocalize with the γ -subunit of AP-1 in Nef-expressing IMR90 cells, we wanted to determine whether Nef was also present at this location. This possibility was suggested previously by the observation that a Nef-GFP chimeric protein stained a perinuclear region in both T cells and fibroblasts, in addition to staining AP-2-containing clathrin coats at the cell surface (Greenberg *et al.*, 1997). The GFP moiety does not alter the normal subcellular distribution of the Nef protein, because the fluorescence pattern of the Nef-GFP chimera colocalized with the distribution of the NA7 Nef protein tagged at its C-terminal end with a short AU1 epitope tag (Goldstein *et al.*, 1992; our unpublished data). Therefore, we compared the distribution of Nef-GFP in the perinuclear region with that of the γ -subunit of AP-1 adaptor complexes, that was detected by indirect immunofluorescence using the 100/3 mAb. As seen in cells A and B in Figure 3, Nef-GFP produced intense fluorescence in the perinuclear regions. This pattern overlapped with the γ -adaptin pattern at this level of resolution (Figure 3, compare panels 1 and 3), consistent with the concentration

of the Nef-GFP chimera in the Golgi. In addition, Nef-GFP was found to clearly colocalize with a subset of γ -adaptin-positive sites (indicated by arrows in Figure 3). However, the localization of Nef-GFP in the Golgi was not limited to γ -adaptin-positive areas, thus indicating a broader distribution of Nef-GFP within the Golgi. The observation that Nef colocalizes with some γ -adaptin-

positive sites in the TGN suggests that Nef may have an active role in recruiting class I MHC complexes to these sites.

Nef promotes retrieval of class I MHC complexes from the plasma membrane to the trans-Golgi

Class I MHC complexes are normally transported to the plasma membrane by the default exocytic pathway (York and Rock, 1996). Class I MHC molecules which accumulate in the *trans*-Golgi could be sorted to this location directly from the exocytic pathway, or following endocytosis from the plasma membrane. To address these possibilities, we asked whether class I MHC complexes internalized from the plasma membrane are transported to the *trans*-Golgi in Nef-expressing cells. To visualize the class I MHC complexes internalized from the surface, transiently-transfected IMR90 cells expressing Nef and the blue fluorescent protein (BFP; Stauber *et al.*, 1998), or BFP alone, were cultured in the presence of the W6/32 mAb. An incubation time of 4 h allowed surface W6/32 mAb complexed with class I MHC to internalize. Cells were then washed, fixed, permeabilized and the distribution of the surface and internalized W6/32 mAb revealed by indirect immunofluorescence with a Texas Red-labeled anti-mouse IgG polyclonal antibody. The remaining class I MHC which had not been recognized during the first incubation, including the class I MHC complexes in the exocytic pathway, were then visualized by staining the cells with fluorescein-conjugated W6/32 mAb. The relationship between the various pools of class I MHC complexes in the cells was assessed by two-color fluorescence microscopy.

As shown in Figure 4, in cells transfected with the control vector, the W6/32 mAb present in the culture medium revealed a fairly uniform distribution of class I MHC complexes throughout the cell (panel 1, W6/32-TxR). This pattern was consistently observed even with cells allowed to internalize the W6/32 mAb for up to 12 h, or stained and then chased in media containing no mAb for an additional 10 h period (our unpublished data). The uniform distribution of class I MHC in IMR90 cells likely reflects the relatively stable expression of class I MHC complexes at the cell surface. In contrast to this uniform pattern, control cells stained with the W6/32-

Fig. 2. Dual-color immunofluorescence microscopy analysis of class I MHC complex localization and Golgi markers in IMR90 cells.

(A) Class I MHC complexes colocalize with γ -adaptin in IMR90 cells expressing Nef. IMR90 fibroblasts transfected with 10 μ g of plasmid expressing NA7 Nef and BFP reporter from the same bicistronic transcription unit were fixed, permeabilized, and various resident Golgi proteins were detected in BFP-positive cells by indirect fluorescence: mannose II was detected with mAb 53FC3 (Burke *et al.*, 1982; Baron *et al.*, 1990) (panel 3), TGN46 was detected with a polyclonal rabbit serum directed against a TGN46 peptide (Prescott *et al.*, 1997) (panel 6), γ -adaptin was detected with mAb 100/3 (Ahle *et al.*, 1988) (panels 9 and 12). The immune complexes were revealed with appropriate polyclonal anti-IgG antibodies labeled with Texas Red. Subsequently, MHC Class I complexes were reacted with mAb W6/32 labeled directly with fluorescein (panels 1, 4, 7 and 10). Panels 10–12 are magnifications of the boxed regions in panels 7–9, respectively. Arrows indicate a subset of vesicles stained for both class I MHC and γ -adaptin. (B) Class I MHC complexes are rarely found in γ -adaptin-positive vesicles in IMR90 cells in the absence of Nef. IMR90 cells were cultured overnight in the presence of 100 U/ml of γ -interferon to stimulate class I MHC expression and the class I MHC complexes and γ -adaptin were visualized as described above.

Fluorescence:

Fig. 3. Localization of the Nef-GFP fusion protein in the Golgi area in IMR90 cells. IMR90 cells transfected with a plasmid expressing NEF-GFP fusion protein were fixed, permeabilized and stained with 100/3 mAb specific for γ -adaptin. Nef-GFP was revealed by direct fluorescence (panel 1) and γ -adaptin by indirect immunofluorescence with a Texas Red-conjugated polyclonal anti-mouse IgG antibody (panel 3). The overlay of the two images is shown in panel 2. Arrows indicate a subset of sites where Nef-GFP and γ -adaptin patterns colocalize.

FITC mAb revealed a concentration of class I MHC in the perinuclear region (Figure 4, panel 2). This class I MHC pool was not labeled by W6/32 mAb internalized from the cell surface and therefore probably represents a concentration of class I MHC complexes in the exocytic pathway.

In cells transfected with the plasmid expressing Nef, the W6/32 mAb present in the culture medium heavily labeled the *trans*-Golgi, but little surface stain was detected (Figure 4, panels 4 and 5). Notably, the distribution of the internalized mAb was similar to that of the remaining (W6/32-FITC mAb-labeled) population of class I MHC complexes in the cells (Figure 4, panel 6). Examination of the magnified images demonstrated colocalization of the two patterns (Figure 4, compare panels 7 and 8). The W6/32 mAb used in this study does not detect free and misfolded class I heavy chain molecules, and therefore did not allow us to address the possibility that some heavy chains may follow a different subcellular route than W6/32-bound class I MHC complexes. Also, we cannot exclude that a fraction of the assembled class I MHC complexes is sorted to the TGN directly from the exocytic pathway, bypassing the cell surface. Nevertheless, our observations indicate that Nef promotes the internalization of class I MHC complexes from the cell surface and, together with the previous observations from localization experiments (Figure 2), that the internalized class I MHC complexes accumulate in a γ -adaptin-positive *trans*-Golgi compartment. Since the internalized W6/32-class I MHC complexes colocalized with the bulk of class I MHC complexes accumulated in the TGN in Nef-expressing cells, it is unlikely that sorting of class I MHC to this location was an artifact induced by the binding of W6/32 mAb. A previous study of T cell lines expressing Nef indicated that class I MHC accumulates in endosomes (Schwartz *et al.*, 1996). We suspect that the rapid flow of class I MHC from the cell surface through the endosomal pathway that occurs in T cells masked the Nef-induced

alteration of class I MHC trafficking in this previous study.

Downregulation of class I MHC and CD4 surface expression involve separable functions of Nef

Involvement of endocytosis in both the downregulation of CD4 and class I MHC expression by Nef (Aiken *et al.*, 1994; Schwartz *et al.*, 1996) suggested that these effects may share similar mechanisms. Therefore we were interested in the relationship between the functions of Nef involved in their downregulation. We showed previously that the ability of Nef to downregulate CD4 involves colocalization of Nef with AP-2-containing clathrin-coated membranes and the ability of Nef to recruit CD4 to these sites (Greenberg *et al.*, 1997). Therefore, we studied the effect of mutations that disrupted the colocalization of Nef with AP-2 on class I MHC downregulation, using a transient expression assay in the CD4-positive JJK subline of human Jurkat T cells (Greenberg *et al.*, 1997; Iafrate *et al.*, 1997).

As shown in Figure 5A, flow-cytometry analysis of JJK T cells transfected with NA7 Nef resulted in an ~50-fold decrease in CD4 expression and 10-fold decrease in class I MHC expression on the surface of positively transfected cells (panel 2). Transfection with plasmids expressing mutant NA7 Nef proteins that do not colocalize with AP-2 complexes [$7_{(174AA)}$ and $7_{(177AAA)}$], or another function of Nef that is possibly required for CD4 recruitment [$7_{(57AAA)}$] (Greenberg *et al.*, 1997), did not affect the ability of these proteins to downregulate class I MHC on the surface of JJK cells (Figure 5A, compare panels 4–6 with 2). Dose-response experiments, which allow a more reliable comparison of wild-type and mutant Nef proteins also revealed that the $7_{(57AAA)}$, $7_{(174AA)}$ and $7_{(177AAA)}$ proteins were wild-type in their ability to downregulate class I MHC (Figure 5B). The observation that the ability of Nef to downregulate class I MHC was unaffected by mutations that disrupt the colocalization of Nef with AP-2 suggests

Fig. 4. The effect of Nef on the distribution of class I MHC complexes internalized from the cell surface in IMR90 cells. IMR90 fibroblasts transfected with 10 μ g of plasmids expressing the BFP marker alone (panels 1–3), or HIV-1 NA7 Nef and the BFP marker from the same bicistronic transcription unit (panels 4–6) were cultured for 4 h in the presence of class I MHC-specific W6/32 mAb. Subsequently, cells were fixed, permeabilized, and the immune complexes were revealed with polyclonal anti-mouse IgG antibody labeled with Texas Red (W6/32 TxR, panels 1, 4 and 7). The unreacted class I MHC complexes in the same cells were revealed with W6/32 mAb labeled directly with fluorescein (W6/32 FITC, panels 2, 5 and 8). The overlay of the two images is shown in panels 3, 6 and 9. Panels 7–9 are magnifications of the boxed regions in panels 4–6.

that Nef uses different mechanisms to induce the endocytosis of class I MHC complexes and of CD4.

Downregulation of class I MHC expression requires the SH3 domain-binding surface and a cluster of acidic amino acid residues in Nef

We asked whether the downregulation of class I MHC maps to surfaces in Nef with other known functional roles. Using a panel of previously characterized mutant Nef proteins (Iafrate *et al.*, 1997), we found that mutations that disrupt the SH3 domain-binding surface of Nef also disrupt class I MHC downregulation. Since these mutations were shown previously to disrupt the ability of Nef to block CD3-initiated signaling in JJK T cells (Iafrate *et al.*, 1997), we studied the relationship between these two effects of Nef.

JJK T cells were transiently transfected with plasmids expressing wild-type or mutant NA7 Nef proteins. To reveal the Nef-induced block in CD3-initiated signaling, cells were stimulated with the mitogenic anti-CD3 mAb HIT-3A. This normally results in rapidly induced expres-

sion of the very early activation marker (CD69) on the cell surface (Yokoyama *et al.*, 1988; Ho *et al.*, 1994). The defective induction of CD69 following anti-CD3 stimulation of Nef-expressing cells provides a convenient indicator of perturbations in CD3-initiated signaling induced by Nef that can be detected by flow-cytometry analysis (Figure 6A, compare panels 2 and 7; Iafrate *et al.*, 1997).

As shown in Figure 6, alanine substitutions for four acidic residues at positions 62–65 [$7_{(62AAA)}$], for prolines P72 and P75 [$7_{(72A,75A)}$], for arginine R77 and lysine K82 [$7_{(77A,82A)}$], and for aspartic acid D86 [$7_{(86A)}$] in Nef disrupted its ability to block CD3-initiated signaling. Interestingly, mutating prolines P72 and P75 also disrupted the downregulation of class I MHC [Figure 6, panel 9, $7_{(72A,75A)}$]. The prolines P72 and P75 are required for the high-affinity-specific interaction of the PPII helix of Nef with the SH3 domain of Hck, and possibly also with other Src-family kinases and SH3 domain-containing proteins (Grzesiek *et al.*, 1996a; Lee *et al.*, 1996; Arold *et al.*, 1997). A similar effect was seen with alanine substitutions

Fig. 5. Flow cytometry analysis of the effect of mutations in Nef on CD4 and class I MHC expression. **(A)** Downregulation of class I MHC complexes and of CD4 expression involve separable functions of Nef. Two-color flow-cytometry analysis of surface class I MHC and CD4 expression in cells transfected with 18 μg of plasmids expressing wild-type (panel 2) or mutant (panels 3–5) Nef proteins, or with a control empty vector (panel 1), and 2 μg of plasmid expressing the CD20 marker. CD4, detected with Leu3A mAb labeled with phycoerythrin, is shown on the ordinate and class I MHC heavy chain, detected with G46 2.6 mAb labeled with fluorescein, is shown on the abscissa. Similar results were obtained using the mAb W6/32 specific for MHC A,B,C heavy chain/β₂-microglobulin complexes. Amino acid substitutions in mutant Nef proteins are indicated above the panels. **(B)** Dose-response analysis of the effect of mutations in Nef on downregulation of CD4 and class I MHC expression. Class I MHC (panel 1), and CD4 (panel 2), expression is shown on the ordinate as peak channel number of CD4, and class I MHC, fluorescence on positively-transfected CD20-positive cells. The amount of vector DNA used for transfection is shown on the abscissa. Class I MHC, CD4 and CD20 were detected simultaneously by three-color flow cytometry (Iafraite *et al.*, 1997).

for R77 and K82 [Figure 6B, 7_(77A,82A)]. These latter residues are also important for the integrity of the SH3-binding surface of Nef. Mutating aspartic acid D86, which was shown to stabilize the Nef-SH3 domain interaction by contacting the RT loop in the SH3 domain (Lee *et al.*, 1996; Arold *et al.*, 1997), also disrupted the ability of Nef to downregulate class I MHC expression. However, dose-response analysis of the effect of this mutation on class I MHC downregulation demonstrated that in contrast to the proline mutations which completely disrupted class I MHC downregulation by Nef, the effect of the D86A mutation was partial (Figure 6B). These observations together indicate that downregulation of class I MHC expression probably involves an interaction of Nef with an SH3 domain-containing protein.

The ability of Nef to downregulate class I MHC expression was also disrupted by mutating the acidic amino acid residues at positions 62–65, which is also important for blocking CD3-initiated signaling by Nef [Figure 6A, panel 3, 7_(62AAAA)]. These residues are not known to be a part of the SH3 domain-binding surface of Nef (Grzesiek *et al.*, 1996; Lee *et al.*, 1996; Arold *et al.*, 1997), suggesting the involvement of additional functions of Nef in downregulating class I MHC expression.

Downregulation of HLA-B7 molecule by Nef requires tyrosine Y320 in the cytoplasmic domain of B7

Class I MHC molecules were shown previously to be endocytosed via clathrin-coated pits (Machy *et al.*, 1987;

Dasgupta *et al.*, 1988), an effect probably mediated by a signal in the cytoplasmic domain of the class I MHC heavy chain molecule (Vega and Strominger, 1989). Therefore, we asked whether deleting the cytoplasmic domain of the class I MHC B7 molecule (Ennis *et al.*, 1990) would prevent its downregulation by Nef. These studies were performed in JJK T cells, which do not express the B7 allele, transiently cotransfected with a bicistronic plasmid expressing B7 and the GFP marker together with a plasmid expressing NA7 Nef, or with a control empty vector. The effect of Nef on the expression of the wild-type and mutant B7 molecules on the cell surface was quantitated by flow-cytometry.

As shown in Figure 7, expression of Nef downregulated the level of wild-type B7 on the cell surface ~10- to 15-fold (B7, compare panels 1 and 4). Deletion of the cytoplasmic domain except for three membrane proximal amino acid residues (R309, R310 and K311) disrupted the effect of Nef, indicating the importance of the cytoplasmic domain (B7.Δ311, Figure 7, compare panels 2 and 5). Since interactions of cytoplasmic domains of endocytosed proteins with AP-2 adaptor complexes frequently involve signals containing tyrosine residues (Ohno *et al.*, 1995; reviewed by Kirchhausen *et al.*, 1997; Marks *et al.*, 1997), alanine was substituted for a single tyrosine Y320 in the B7 cytoplasmic domain (B7.Y320A), which is conserved in other class I MHC A and B alleles. This substitution disrupted the effect of Nef on B7 expression (Figure 7, compare panels 3 and 6), suggesting that Nef may modulate

Fig. 6. Flow cytometry analysis of the effect of mutations in the SH3 domain-binding surface of Nef on the downregulation of class I MHC and CD3-initiated signaling. **(A)** The effect of mutations in Nef on class I MHC and CD69 expression. Cells were transfected with 18 μg plasmids expressing NA7 Nef (panels 2 and 7), mutant Nef proteins (panels 3–5 and 8–10), or a control empty vector (panels 1 and 6), and 2 μg of plasmid expressing the CD20 marker and cultured overnight in the absence (Mock, panels 1–5), or presence (anti-CD3, 200 ng/ml, panels 6–10) of HIT-3A anti-CD3 mAb. **(B)** Dose-response analysis of the effect of mutations in Nef on class I MHC expression. Class I MHC expression is shown on the ordinate as peak channel number of class I MHC fluorescence on positively transfected CD20-positive cells. The amount of vector DNA used for transfection is shown on the abscissa. Class I MHC and CD20 were detected simultaneously by two-color flow cytometry.

the interaction of B7, and other class I MHC molecules, with the endocytic and/or sorting machineries via this tyrosine. Since tyrosines are targets of signal transduction pathways (reviewed by Hunter, 1987), the requirement of a tyrosine residue in the class I MHC cytoplasmic domain potentially links class I MHC downregulation by Nef to the signal transduction machinery.

Nef-induced accumulation of class I MHC complexes in the trans-Golgi requires the SH3 domain-binding surface and a cluster of acidic amino acid residues in Nef

To confirm that the accumulation of class I MHC complexes in the *trans*-Golgi is a part of the mechanism for class I MHC downregulation by Nef, we studied the effects of mutations in Nef that disrupt class I MHC downregulation in JJK T cells on the trafficking of class I MHC complexes in IMR90 cells. As shown in Figure 8, two mutations that disrupted class I MHC downregulation in JJK T cells also disrupted the internalization of class I MHC and its accumulation in the *trans*-Golgi in IMR90 cells [panels 3 and 4, 7_(62AAAA), and 9 and 10, 7_(72A,75A)]. Mutating aspartic acid D86 had little detectable effect on the accumulation of class I MHC in the *trans*-Golgi in IMR90 cells (Figure 8, panels 5 and 11), even though this mutation partially disrupted class I MHC downregulation in JJK T cells. However, this difference is probably explained by the fact that the class I MHC localization assays in IMR90 cells are not quantitative, making the

detection of a partial loss of function difficult. In contrast, a number of other mutations in Nef that did not affect class I MHC downregulation in JJK cells also had no effect on the ability of Nef to promote intracellular accumulation of class I MHC in IMR90 cells [e.g. Figure 8, panels 6 and 12, 7_(177AAA)]. The similar genetic requirements for the downregulation of class I MHC in T cells and for the accumulation of internalized class I MHC complexes in the *trans*-Golgi in fibroblasts suggests that Nef perturbs class I MHC trafficking in these cell types similarly. Furthermore, these data directly demonstrate that the SH3 domain-binding surface and a cluster of acidic amino acid residues are required for the internalization of class I MHC complexes and their accumulation in the *trans*-Golgi.

Discussion

Our studies indicate that Nef downregulates class I MHC by promoting the accumulation of class I MHC complexes, which have been internalized from the plasma membrane, in AP-1 containing regions in the *trans*-Golgi. Interestingly, we found that the molecular interactions between Nef and the protein sorting machinery that are required for class I MHC downregulation are different from those required for CD4 downregulation. Mutations in Nef that disrupt its colocalization with AP-2-containing clathrin coats at the cell surface, thereby abolishing the ability of Nef to downregulate CD4 surface expression (Greenberg

Fig. 7. Effect of mutations in the cytoplasmic domain of class I MHC B7 heavy chain on its downregulation by Nef. JJK T cells were cotransfected with 10 μ g of plasmid expressing the wild-type (panels 1 and 4) or mutant (panels 2, 3, 5 and 6) B7 heavy chain molecules and the GFP marker from the same bicistronic transcription unit, and with 10 μ g of NA7 Nef expression vector (Nef, panels 4–6) or of a control empty vector (Mock, panels 1–3). Histograms of B7 heavy chain expression on the cell surface are shown for populations of cells with identical levels of GFP expression.

et al., 1997), do not affect the downregulation of class I MHC surface expression. Instead, class I MHC downregulation requires a cluster of acidic amino acid residues, as well as the residues that constitute the SH3 domain-binding surface, in Nef. Notably, both of these elements are dispensable for the ability of Nef to downregulate cell surface expression of CD4, but are required for the ability of Nef to block CD3-initiated signaling (Iafrate *et al.*, 1997). These results are provocative since they link the endocytic and signal transduction machineries in the regulation of class I MHC surface expression, which possibly occurs via its conserved cytoplasmic domain tyrosine residue. Our observations suggest a novel mechanism for the downregulation of class I MHC surface expression, where Nef regulates the interaction of a tyrosine-based endocytic motif in class I MHC molecules with the endocytic machinery.

Previous studies suggested that the rates of class I MHC turnover in T cells and macrophages are more rapid than those found with other cell types (Machy *et al.*, 1987; York and Rock, 1996), thus indicating that aspects of the metabolism and sorting of class I MHC molecules are probably regulated in a cell type-specific manner. Interestingly, we found that the amino acid residues in Nef that are required for the downregulation of class I MHC complexes in T cells are also required for their accumulation in the TGN in fibroblasts. This correlation implies that these effects of Nef on class I MHC complexes in T cells and in fibroblasts involve the same surfaces of Nef and provide strong evidence that Nef targets a molecular mechanism regulating expression of class I MHC complexes that is common for both cell types.

The observation that the ability of Nef to colocalize with AP-2-containing clathrin coats is dispensable for class I MHC downregulation is intriguing because the constitutive endocytosis of class I MHC was reported to involve clathrin coated pits and because the Nef-induced endocytosis of class I MHC complexes involves amino acid residues that resemble endocytosis signals normally recognized by the AP-2 adaptor complex (York and Rock, 1996). Therefore, unlike CD4 downregulation, class I MHC downregulation is not likely to be mediated by direct recruitment of class I MHC molecules by Nef into the endocytic pathway at clathrin-coated regions at the plasma membrane. We could not detect interactions between HIV-1 Nef, the cytoplasmic domain of class I

Fig. 8. Effect of mutations in Nef on the internalization and distribution of class I MHC complexes in IMR90 cells. IMR90 fibroblasts transfected with 10 μ g of plasmids expressing the wild-type (panels 2, 8, 14) or mutant (panels 3–6, 9–12, 15–18) NA7 Nef proteins and the BFP marker from the same bicistronic transcription unit, were allowed to internalize class I MHC-specific W6/32 mAb for 4 h and the immune complexes were revealed as described in Figure 4 (W6/32 TxR, panels 1–6). The unreacted class I MHC complexes in the same cells were revealed with W6/32 mAb labeled directly with fluorescein (W6/32 FITC, panels 7–12). The overlay of the two images is shown in panels 13–18.

B7 heavy chain, and subunits of the AP-1 and AP-2 adaptor protein complexes using the yeast two-hybrid system (A.J.Iafrate, T.Swigut and J.Skowronski, unpublished data). These data suggest that Nef may promote class I MHC endocytosis and accumulation of the endocytosed class I MHC complexes in the *trans*-Golgi indirectly. Since AP-1-containing clathrin-coated vesicles mediate traffic from the *trans*-Golgi to endosomes *en route* to lysosomes (Traub and Kornfeld, 1997), class I MHC molecules in the *trans*-Golgi are probably targeted for degradation.

The effect of Nef on the endocytosis of CD4 and class I MHC complexes involves different amino acid residues in their cytoplasmic domains: a dileucine motif in CD4 and a tyrosine in class I MHC heavy chain, exemplified by Y320 in the B7 molecule. Dileucine and tyrosine-

based motifs are two different classes of signals that direct sorting of transmembrane proteins via interactions with the AP-2 adaptor protein complex at the plasma membrane and via other adaptor protein complexes such as AP-1, and probably AP-3, at the Golgi and other locations (reviewed by Kirchhausen *et al.*, 1997; Marks *et al.*, 1997). Therefore, it is not surprising that the effect of Nef on CD4 and class I MHC endocytosis involves different sets of molecular interactions and different mechanisms. Nef could regulate the interaction of the tyrosine-based endocytic motif in class I MHC with the endocytic machinery by altering the phosphorylation of this tyrosine. This is supported by a recent observation that the tyrosine-based sorting motif in CTLA-4 is regulated by tyrosine phosphorylation (Shiratori *et al.*, 1997). Since class I MHC downregulation involves residues in Nef known to be important for altering signal transduction pathways, and specifically those interacting with Src-family tyrosine kinases, this model is especially attractive.

The involvement of a Src-family kinase is suggested by the observation that the ability of Nef to downregulate class I MHC is disrupted by a number of mutations at the residues that form the surface of Nef that binds the SH3 domains of Fyn and Hck (Grzesiek *et al.*, 1996a; Lee *et al.*, 1996; Arold *et al.*, 1997). This surface includes prolines P72 and P75 of the PPII helix, which directly contact the SH3 domain cores, or arginine R77, lysine K82, and aspartic acid D86, which are critical for the overall integrity of the SH3 domain-binding surface. Interestingly, tyrosine Y320 in the cytoplasmic domain of class I B7 molecule was shown previously to be a substrate for Src kinase *in vitro* (Guild *et al.*, 1983). While these data suggest that the internalization of class I MHC complexes could be mediated by a Nef–Src-family kinase complex, such a simplistic model is not likely to be correct because overexpression of wild-type or constitutively active forms of Fyn, Lck or Hck inhibit, rather than promote class I MHC downregulation by Nef (our unpublished data). Therefore, it is more likely that a Nef–Src-kinase complex could indirectly regulate class I MHC internalization and/or accumulation in the *trans*-Golgi. Alternatively, the SH3 domain-binding surface of Nef may be involved in interactions with other proteins that connect the endocytic and signal transduction machineries. The observation that an alanine substitution for aspartic acid D86 in Nef, predicted to destabilize the interaction of Nef with SH3 domains of Src-family kinases (Lee *et al.*, 1996; Arold *et al.*, 1997), only partially disrupts downregulation of class I MHC complexes is consistent with this possibility.

The ability of Nef to downregulate class I MHC expression also requires a cluster of acidic amino acid residues conserved in HIV-1 Nef (Shugars *et al.*, 1993). This acidic element is not known to be a part of the surface of Nef that mediates interactions with SH3 domains of Src-family kinases (Grzesiek *et al.*, 1996; Lee *et al.*, 1996), suggesting that interactions of Nef with a non-SH3 domain-containing protein is also important for the downregulation of class I MHC expression. Although we do not know a candidate protein for this interaction, the acidic element in Nef may function as a sorting signal because a similar cluster of acidic amino acids in the cytoplasmic domain of furin, a *trans*-Golgi protein that

cycles between the *trans*-Golgi and the plasma membrane, mediates its retrieval from the cell surface and its accumulation in the *trans*-Golgi (Jones *et al.*, 1995; Schafer *et al.*, 1995; Voorhees *et al.*, 1995). Also, a similar acidic cluster is part of a signal in the cytoplasmic domain of the mannose 6-phosphate receptor, and varicella zoster virus glycoprotein I (VZV-gpI), that mediates the recruitment of AP-1 adaptor complexes to *trans*-Golgi membranes (Le Borgne *et al.*, 1993; Alconada *et al.*, 1996; Mauxion *et al.*, 1996). This evidence suggests a model whereby the acidic element in Nef targets a Nef–class I MHC complex, that has been endocytosed from the plasma membrane, to the *trans*-Golgi. Alternatively, since the acidic element is required for the effect of Nef on signal transduction, this sequence may have additional roles in class I MHC downregulation.

Downregulation of class I MHC has been exploited by many viruses as a means of evading the anti-viral immune response of the infected host. Most frequently viral gene products interfere with the assembly of functional class I MHC complexes in the endoplasmic reticulum, or their transport along the exocytic pathway (for reviews see Fruh *et al.*, 1997; Wiertz *et al.*, 1997). The mechanism for the downregulation of class I MHC expression by Nef, which involves redirecting the traffic of the internalized class I MHC complexes is unusual and its advantage over those used by other viral gene products is not immediately obvious. It is possible that such a mechanism is especially effective in T cells and macrophages, the cell types targeted by immunodeficiency viruses. Since the turnover of class I MHC complexes in these cell types is rapid, a viral protein could efficiently downregulate class I MHC surface expression in these cell types by preventing their recycling to the cell surface. Nef could thereby prevent presentation of peptides acquired from the endocytic pathway (reviewed by Rock, 1996). It is also possible that the mechanism used by Nef to downregulate class I MHC complexes may have evolved in the context of the other molecular interactions of Nef with the protein sorting and signal transduction machineries required for its other diverse functions.

Materials and methods

Plasmid construction

The oligonucleotide-directed site-specific mutagenesis of NA7 Nef, a natural HIV-1 *nef* allele, has been described previously (Mariani and Skowronski, 1993; Iafrate *et al.*, 1997). Mutated *nef* sequences were verified by DNA sequencing and subcloned between the *Xba*I and *Bam*HI sites of a T cell-specific pCD3- β , or the CMV-based pCG, expression vector, as described previously (Tanaka and Herr, 1990; Skowronski *et al.*, 1993). The bicistronic expression vectors either contain NA7 Nef and GFP, or BFP, under translational control of the EMCV IRES element isolated from pCITE2 vector (Novagen), or contain the class I MHC B7 heavy chain gene (Ennis *et al.*, 1990) followed by the IRES GFP cassette, were constructed using PCR and standard subcloning techniques (Sambrook *et al.*, 1989), and subcloned into the pCG expression plasmid. Genes expressing the GFP and BFP proteins were kindly provided by George N.Pavakis (Stauber *et al.*, 1998). The design of NA7.GFP fusion protein was described previously (Greenberg *et al.*, 1997).

Cell lines and DNA transfections

Jurkat T cells (JJK subline) expressing high levels of human CD4, provided by Dan R.Littman, were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES (pH 7.4) and 10% fetal bovine serum (FBS), and the cultures were diluted 1:10 to 1:20

every 3–4 days (Iafrate *et al.*, 1997). Human fibroblasts (IMR90) were grown in DME medium supplemented with 10% FBS and the cultures were diluted 1:4 following trypsinization every 3–4 days. JJK cells from exponentially growing cultures, or subconfluent IMR90 cells were transfected by electroporation as described previously (Greenberg *et al.*, 1997; Iafrate *et al.*, 1997). Briefly, cells were electroporated at 200 V and 960 μ F with a total of 10 μ g to 20 μ g DNA containing varying amounts of the appropriate expression vectors, and 2 μ g of CMV CD20 expression plasmid for use as a marker of transfected cells in flow-cytometry. The transfected cells were cultured for an additional 15–36 h prior to flow-cytometric and/or microscopic analyses of class I MHC and CD20 reporter levels, and/or class I MHC and GFP expression.

Flow-cytometry analysis

The effect of Nef on surface expression of class I MHC in transiently transfected cells was analyzed on an Epics-Elite flow-cytometer, as described previously (Iafrate *et al.*, 1997). Briefly, aliquots of 2×10^5 cells were suspended in phosphate-buffered saline (PBS) containing 1% FBS and 0.1% sodium azide (PBS-FA) and stained with saturating amounts of phycoerythrin-conjugated Leu 3A mAb, specific for CD4 and fluorescein (FITC)-conjugated G46-2.6 mAb, specific for class I MHC heavy chain (Pharmingen), or FITC-conjugated W6/32 mAb specific for the assembled class I MHC heavy chain β_2 -microglobulin complex (Sigma). Alternatively, biotinylated B9.12.1 specific for class I MHC heavy chain in assembled complex with β_2 -microglobulin (Malissen *et al.*, 1982; Immunotech) followed by phycoerythrin-conjugated streptavidin (Caltag) together with FITC-conjugated FN50 mAb which recognizes CD69, was used. In both cases PerCP-conjugated Leu-16 mAb, specific for CD20 (Becton and Dickinson), was used. For dose-response analysis, the level of CD4 or class I MHC expression on the surface of CD20-positive cells, represented by peak channel number of green or red fluorescence, was measured as a function of the amount of Nef-expression vector DNA used for transfections (Skowronski and Mariani, 1995). For the effect of Nef on B7, cells transfected with the bicistronic transcription unit expressing B7 and GFP were incubated with mAb BB7.1 (Brodsky *et al.*, 1979; Pharmingen) followed by Tricolor-conjugated anti-mouse IgG (Caltag) and surface B7 levels detected as a function of green fluorescence by flow-cytometry.

Immunofluorescence microscopy analysis

Transfected IMR 90 fibroblasts were plated and grown on glass coverslips. At 15–36 h post-transfection, the cells were fixed in 3% paraformaldehyde for 20 min at room temperature and permeabilized in 0.1% NP40 in PBS. Cells were then incubated for 30 min in blocking solution (3% BSA, 0.1% NP40 in PBS) followed by incubation with the appropriate antibodies in blocking solution. Steady-state class I MHC levels were visualized in Nef-expressing cells, identified by green or blue fluorescence produced upon transfection with the bicistronic Nef and GFP/BFP expression vectors, by incubation with mAb W6/32 recognizing assembled human class I MHC (Barnstable *et al.*, 1987; Jackson *et al.*, 1992), followed by staining with Texas Red-conjugated anti-mouse IgG (Amersham). In localization experiments, transfected cells were first incubated with 53FC3 mAb, which reacts with anti-mannosidase II (Burke *et al.*, 1982; Baron *et al.*, 1990), or with 100/3 mAb, which recognizes γ -adaptin (Ahle *et al.*, 1988; Sigma), or anti-TGN46 P12 serum (Prescott *et al.*, 1997), followed by incubation with either Texas Red-conjugated anti-mouse for 100/3 and for 53FC3 mAbs, or anti-rabbit IgG (Amersham) for anti-TGN46 serum. Subsequently, cells were blocked with 5% normal mouse serum and stained with FITC-conjugated W6/32 (Sigma). For internalization experiments, transfected cells were co-cultured with non-conjugated W6/32 (Accurate) for 4 h at 37°C, fixed, permeabilized and reacted with Texas Red-conjugated anti-mouse IgG to detect internalized class I MHC complexes. Cells were then blocked with 5% normal mouse serum and stained with FITC-conjugated W6/32 to reveal unreacted class I MHC molecules. Coverslips were washed in PBS before mounting onto glass slides in glycerol based mounting media. Fluorescence microscopy images were taken with a Nikon Microphot-FXN microscope equipped with a CCD camera and processed using Oncor Imaging software.

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