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HIV-1 Nef Assembles a Src Family Kinase-ZAP-70/Syk-PI3K Cascade to Downregulate Cell-Surface MHC-I

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SUMMARY

HIV-1 Nef, which is required for the efficient onset of AIDS, enhances viral replication and infectivity by exerting multiple effects on infected cells. Nef downregulates cell-surface MHC-I molecules by an uncharacterized PI3K pathway requiring the actions of two Nef motifs—$EEEE_{65}$ and $PXXP_{75}$. We report that the Nef $EEEE_{65}$ targeting motif enables Nef $PXXP_{75}$ to bind and activate a *trans*-Golgi network-localized Src family tyrosine kinase (SFK). The Nef/SFK complex then recruits and phosphorylates the tyrosine kinase ZAP-70, which binds class I PI3K to trigger MHC-I downregulation in primary $CD4^+$ T cells. In promonocytic cells, Nef/SFK recruits the ZAP-70 homolog Syk to downregulate MHC-I, implicating this PI3K pathway in multiple HIV-1 reservoirs. Isoform-specific PI3K inhibitors repress MHC-I downregulation, identifying them as potential therapeutic agents to combat HIV-1. The discovery of this Nef-SFK-ZAP-70/Syk-PI3K signaling pathway explains the hierarchal role of the Nef motifs in effecting immunoevasion.

INTRODUCTION

In infected people, HIV-1 establishes long-lived reservoirs in a number of cell types, including macrophages, dendritic cells, and resting $CD4^+$ T cells, which resist highly active antiretroviral therapy (HAART; Stevenson, 2003). To respond to the virus infection, the host activates an antiviral response, integrating adaptive immunity and apoptotic mechanisms to destroy the virus. Pathogenic viruses counter the host antiviral response by expressing specialized genes that prevent antigen presentation and apoptosis (Benedict et al., 2002; Peterlin and Trono, 2003). Unlike other pathogenic viruses, HIV-1 uses a limited set of gene products to coordinate the antiviral counterattack. One of these gene products, Nef, is a 27 kDa N-myristoylated protein that enhances viral replication and virion infectivity and is required for the onset of AIDS following HIV-1-infection (Das and Jameel, 2005; Peterlin and Trono, 2003). Nef affects cells in many ways, including altering T cell activation and maturation (Stevenson, 2003; Stove et al., 2003; Thoulouze et al., 2006), subverting the apoptotic machinery, and downregulating CD4 molecules and major histocompatibility complex class I (MHC-I) molecules encoded by the HLA-A and -B loci (Peterlin and Trono, 2003). The downregulation of MHC-I by SIV Nef in rhesus macaques limits $CD8^+$ T cell-mediated killing and contributes to the pathogenic effect of Nef, illustrating the importance of Nef-mediated immunoevasion to disease progression (Swigut et al., 2004).

Current HIV-1 therapeutics principally target the activities of virally encoded reverse transcriptase and protease. However, their effectiveness is compromised by the emergence of drug-resistant viral strains. A promising alternative approach is to develop therapeutics that interfere with the action of HIV-1 proteins on cellular factors (Greene, 2004). HIV-1 Nef represents a potential target for such an approach, as it binds to and stimulates the activity of several cellular kinases, including Src family tyrosine kinases (SFKs; Lee et al., 1995; Trible et al., 2006), which promotes HIV-1 disease in animal models (Hanna et al., 2001), and class I PI3K, which enables HIV-1 to increase virus production, block apoptosis, and downregulate cell-surface MHC-I (Blagoveshchenskaya et al., 2002; Linnemann et al., 2002; Peterlin and Trono, 2003). The profound ability of the Bcr-Abl and c-kit inhibitor Gleevec to cure specific cancers supports such an approach (Druker, 2004). Unfortunately, current PI3K inhibitors, including wortmannin and LY294002, are panselective, showing a similar IC_{50} against all PI3Ks (Ward et al., 2003). Moreover, the concentrations required for LY294002 to block PI3K are similar to the concentrations

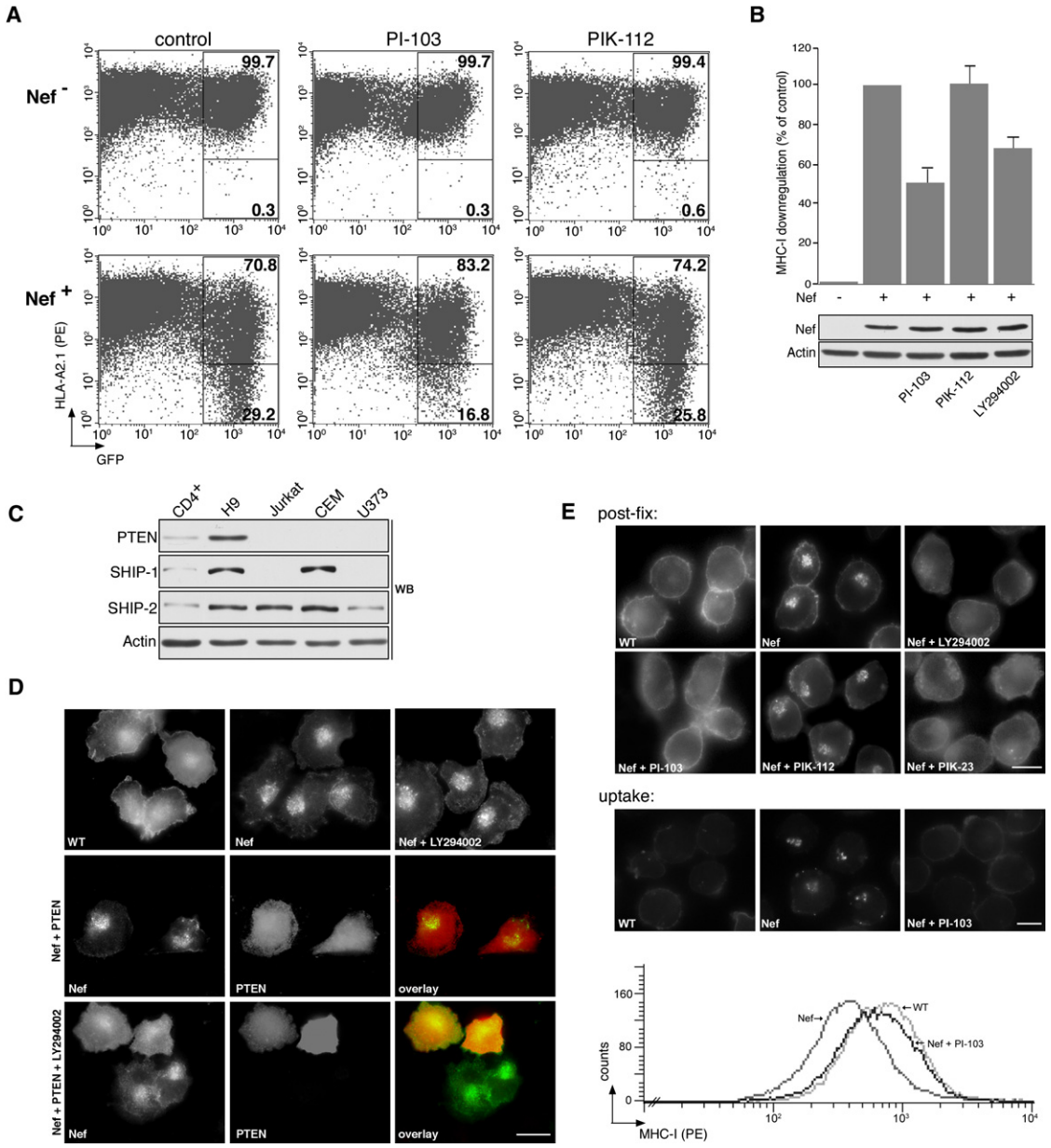

Figure 1. Nef-Triggered MHC-I Downregulation in Primary CD4+ T Cells Is Mediated by Class I PI3K

(A) Primary CD4+ T cells incubated with 2 ng/ml IL-7 for 4 days were infected with the Nef− or Nef+ pseudotyped viruses (moi = 5). At 40 hr postinfection cells were treated with 1 μM PI-103, 1 μM PIK-112, or 1% DMSO (control) for 3 hr. The cells were then incubated with mouse-anti-HLA-A2.1 followed by anti-mouse-PE-conjugated Ig and analyzed by flow cytometry. Viable cells were analyzed for eGFP and anti-HLA-A2.1. The frequency of eGFP^{high} cells displaying downregulated MHC-I is shown in the lower right gate. Similar results were obtained using PHA/IL-2-stimulated primary CD4+ T cells or using mAb W6/32 (data not shown). Western blot analysis showed that Nef expression was greater in eGFP^{high}/MHC-I^{low} cells than in eGFP^{high}/MHC-I^{high} cells, revealing an incomplete correlation between Nef and GFP expression in this vector (data not shown).

(B) Nef− or Nef+ pseudovirus-infected primary CD4+ T cells were treated with 1 μM PI-103, 1 μM PIK-112, 5 μM LY294002, or 1% DMSO for 3 hr and then analyzed by flow cytometry. The effect of each compound on MHC-I downregulation was then normalized to the extent of MHC-I downregulation in control cells infected with the Nef+ or Nef− pseudoviruses (set at 100 and 1, respectively). Bottom: Western blot showing the expression of Nef and the levels of actin (input). Error bars represent the mean ± SD of four independent experiments with cells isolated from three donors (n = 4).

(C) Primary CD4+ T cells or the indicated cell lines were harvested, and the expression of the indicated proteins was determined by western blot.

(D) U373 MG cells were transfected or not with pSG5-PTEN-HA for 48 hr and then infected with VV:WT or VV:Nef (moi =10, 4 hr). Where indicated, cells were treated with 5 μM LY294002 for 40 min prior to fixation. The cells were then fixed and stained with anti-MHC-I (mAb W6/32, green) and anti-HA (red). Scale bar, 20 μm.

(E) Top: H9 CD4+ T cells were infected with VV:WT or VV:Nef (moi = 10, 4 hr) and then treated or not with PI-103 (1 μM), PIK-112 (1 μM), or LY294002 (5 μM) for 1 hr. Cells were fixed and MHC-I molecules were stained with mAb W6/32. Scale bar, 10 μm. Middle: H9 CD4+ T cells infected with VV:WT

that cause cell death—precluding their use as therapeutics (Ward et al., 2003). Recently, a new group of small-molecule PI3K inhibitors were developed that are selective for the class I PI3K isoforms (Knight et al., 2006). One of these inhibitors, PI-103, which is a pyridinylfuranopyrimidine derivative, targets multiple class I p110 catalytic subunits, blocking PKB/Akt activation and arresting cells in G_0/G_1 without the toxicity associated with panselective PI3K inhibitors (Fan et al., 2006; Knight et al., 2006).

The development of isoform-specific PI3K inhibitors suggests a novel approach to combat HIV-1. However, the role of PI3K in Nef-mediated MHC-I downregulation is controversial. We reported that Nef diverts cell-surface MHC-I molecules to *trans*-Golgi network (TGN)-associated compartments in heterologous cells by a PI3K-stimulted, ARF6-dependent, endocytic pathway (Blagoveshchenskaya et al., 2002). This MHC-I downregulation requires the hierarchical action of three motifs (Das and Jameel, 2005; Peterlin and Trono, 2003): an acidic cluster ($EEEE_{65}$), required for binding to the cytosolic sorting protein PACS-1 (Piguet et al., 2000); an SH3 domain-binding motif ($PQVP_{75}$) that directs association of Nef with SFKs (Lee et al., 1995); and an N-proximal α-helical region containing a critical methionine (M_{20}), which promotes association of MHC-I with the heterotetrameric sorting adaptor AP-1 (Roeth et al., 2004). The conservation of these three motifs in the pandemic M group HIV-1, which accounts for over 90% of all AIDS cases worldwide, suggests that they control an essential pathway required for HIV-1 pathogenesis (Keele et al., 2006). But this model has been challenged by others who reported that in leukemic T cell lines or in U373 astrocytoma cell lines Nef acts solely on newly synthesized MHC-I molecules and not on cell-surface MHC-I, and that Nef acts independently of PI3K because the panselective PI3K inhibitors LY294002 or wortmannin failed to block MHC-I downregulation in these transformed cell lines (Kasper and Collins, 2003; Larsen et al., 2004).

We sought to determine the basis for the conflicting models of MHC-I downregulation, and in doing so, we discovered a PI3K activation pathway used by HIV-1 Nef to downregulate cell-surface MHC-I in HIV-1 target cells. We show that the Nef $EEEE_{65}$ targeting motif, which is required for efficient binding to PACS-1 (Piguet et al., 2000), enables the Nef $PXXP_{75}$ motif to bind and activate an SFK localized to TGN-associated reservoirs. The Nef/SFK complex then recruits and phosphorylates ZAP-70, which activates Nef-associated PI3K to trigger the PI-103-sensitive downregulation of MHC-I in primary $CD4^+$ T cells and model $CD4^+$ T cell lines. We also show that, in promonocytic cells and heterologous cell types, Nef/SFK recruits the ZAP-70 homolog Syk to stimulate the PI3K-dependent downregulation of cell-surface MHC-I. Our elucidation of this Nef-SFK-ZAP-70/Syk-PI3K signaling pathway explains the hierarchal role of the Nef motifs that control immunoevasion and identifies new targets for HIV-1 therapy with the potential to make use of newly developed isoform-specific PI3K inhibitors.

RESULTS

HIV-1 Nef Uses a PI3K-Dependent Pathway to Downregulate Cell-Surface MHC-I in $CD4^+$ T Cells

HIV-1 Nef utilizes PI3K to downregulate cell-surface MHC-I in heterologous cell types, but whether it uses this same pathway to downregulate MHC-I in HIV-1 target cells and the mechanism controlling this pathway are unknown. We thus tested the effect of PI3K inhibitors on Nef-induced MHC-I downregulation in primary $CD4^+$ T cells isolated from healthy donors. Cells were pretreated with PHA/IL-2 or IL-7 and then infected with VSV-G pseudotyped, GFP-expressing HIV-1 viruses derived from HIV-1 NL4-3 that either lack the Nef gene or express Nef (Husain et al., 2002). Whereas PHA/IL-2 robustly stimulates cellular PI3K activity and T cell activation, nonmitogenic levels of IL-7 used here do not (Figure S1 in the Supplemental Data available with this article online). We then determined the frequency of GFP-positive infected cells with downregulated cell-surface HLA-A2.1 by flow cytometry. Like the activity of other Nef alleles (Keppler et al., 2006), NL4-3 Nef downregulated cell-surface HLA-A2.1 by 40%–60% as determined by fluorescence intensity regardless of treatment with IL-7 or IL-2/PHA (Figures 1A and 1B and data not shown). Parallel cultures were treated with the class I PI3K inhibitor PI-103 or its inactive analog, PIK-112, for 3 hr prior to analysis, with no change in cell viability as determined by forward and side scattering. We found that PI-103 inhibited the Nef-induced MHC-I downregulation in primary $CD4^+$ T cells, whereas PIK-112 had no effect (Figure 1A), suggesting that Nef uses a PI3K-dependent pathway to efficiently downregulate cell-surface MHC-I in primary $CD4^+$ T cells. By contrast, PI-103 had no measurable effect on Nef-mediated CD4 downregulation (Figure S2). Moreover, the inhibition of MHC-I downregulation by PI-103 was similar to that observed with the commonly used, panselective PI3K inhibitor LY294002, which also inhibits MHC-I downregulation in heterologous cells (Figure 1B).

The ability of PI3K inhibitors to repress efficient downregulation of cell-surface MHC-I by HIV-1 Nef in primary $CD4^+$ T cells was in direct conflict with other reports. PI3K activity had no effect on Nef-mediated downregulation of MHC-I in the leukemic T cell lines Jurkat and CEM or in U373 cells (Kasper and Collins, 2003; Larsen et al., 2004). However, PIP_3, the product of the class I PI3Ks, is rapidly dephosphorylated by one of several D-3 (PTEN) or D-5 (SHIP-1 and -2) lipid phosphatases, attenuating PI3K-stimulated signaling pathways (Deane and Fruman, 2004). Interestingly, Jurkat, CEM, and U373 cell lines

or VV:Nef and treated or not with PI-103 as above were incubated with mAb W6/32 (3 μg/ml) for 30 min and then chased for an additional 30 min, fixed, and processed for immunofluorescence microscopy. Scale bar, 10 μm. Bottom: H9 $CD4^+$ T cells were infected with VV:WT or VV:Nef (moi = 10, 8 hr), then treated or not with PI-103 for 1 hr, and then analyzed by flow cytometry using mAb W6/32.

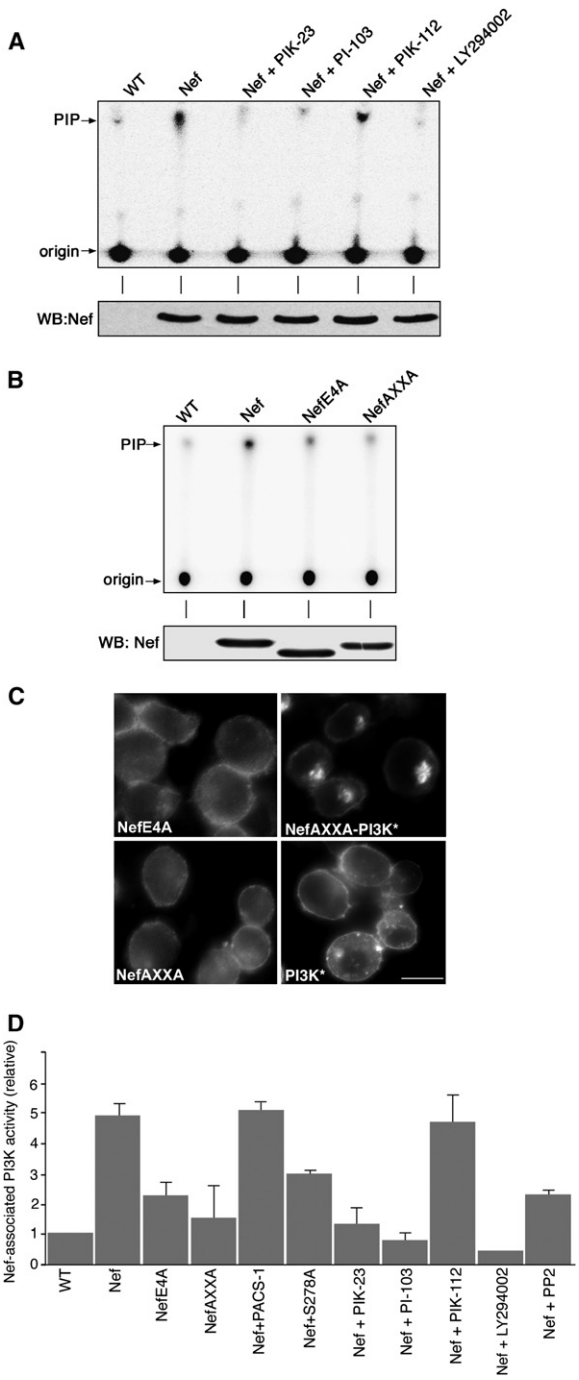

Figure 2. Nef-Stimulated PI3K Activity Requires Nef $EEEE_{65}$ and $PXXP_{75}$

(A) Primary $CD4^+$ T cells cultured in Il-7 were infected with VV:WT or VV:Nef/f (moi = 10, 12 hr). Following Nef/f immunoprecipitation (shown on western blot), the samples were treated or not with 0.1 μM PIK-23, PI-103, or PIK-112 or with 10 μM LY294002 or DMSO for 10 min, and Nef-associated PI3K activity was measured as described in the Experimental Procedures.

(B) H9 $CD4^+$ T cells infected with the indicated VV vectors (moi = 5, 8 hr) were lysed, and Nef-associated PI3K was measured as described in the Experimental Procedures. Bottom: Western blot showing the expression of Nef/f constructs.

(C) H9 $CD4^+$T cells expressing the indicated constructs were processed for immunofluorescence microscopy, and MHC-I molecules were detected with mAb W6/32 as described in the legend to Figure 1E. Scale bar, 10 μm.

(D) Replicate plates of H9 $CD4^+$ T cells infected with VV:WT or with VV recombinants expressing the indicated proteins (total moi = 5, 8 hr) were treated or not with 10 mM PP2 or, following immunoprecipitation with mAb M2, with the indicated PI3K inhibitors as described in (A) and then analyzed for PI3K activity. Error bars represent the mean ± SD of three independent experiments.

lack PTEN (Figure 1C). Consistent with the absence of PTEN, Jurkat cells contain inordinately high levels of PIP_3, an otherwise low-abundance and transiently produced phosphoinositide (Astoul et al., 2001). Therefore, we speculated that, in the absence of a PIP_3 phosphatase, transient inhibition of PI3K activity would marginally affect PIP_3 levels, thus negating the pharmacologic inhibition of PI3K-dependent HIV-1 Nef-mediated MHC-I downregulation. To test this possibility, we expressed HIV-1 Nef in U373 cells in the absence or presence of LY294002, and in agreement with others (Larsen et al., 2004), we found that the inhibitor had no effect on MHC-I downregulation (Figure 1D). We then asked whether rescue of PTEN expression in U373 cells would restore sensitivity of the Nef-mediated MHC-I downregulation pathway to LY294002. Accordingly, we found that LY294002 inhibited Nef-mediated MHC-I downregulation in PTEN-rescued U373 cells (Figure 1D).

Our determination that HIV-1 Nef requires a PI3K in U373 cells led us to ask whether PI3K activity is required for Nef to downregulate cell-surface MHC-I in H9 $CD4^+$ T cells, which like primary $CD4^+$ T cells are replete with expression of PTEN and other PIP_3 phosphatases (Figure 1C). Accordingly, the panselective inhibitor LY294002 and the class I PI3K inhibitor PI-103 hindered Nef-mediated downregulation of MHC-I in H9 cells (Figure 1E), but not in cells treated with PTEN siRNA (Figure S3). Because PI-103 inhibits mTOR and class I p110 catalytic subunits, we tested whether PIK-23, a quinazolinone purine derivative that selectively targets p110δ (Knight et al., 2006), could inhibit MHC-I downregulation. Like PI-103, PIK-23 repressed Nef-mediated MHC-I downregulation in H9 $CD4^+$ T cells at all concentrations tested (Figure 1E). The PI-103-sensitive, Nef-induced redistribution of MHC-I to the paranuclear region resulted primarily from the downregulation of cell-surface molecules as determined by antibody uptake and flow cytometry (Figure 1E), as well as by the inability of Nef to block delivery of newly synthesized MHC-I molecules to the cell surface (Figure S4). Control experiments showed that the amount of Nef expression per infected cell using either the vaccinia or pseudovirus vectors did not exceed the amount of Nef expressed in HIV-1-infected cells (Figure S5), supporting the physiologic relevance of these results. Furthermore, expression of mutant proteins that block the ARF6-dependent endocytic pathway—including $ARNOE_{156}K$, an inactive form of the PIP_3 binding ARF6 GEF, ARNO, and the ARF6 mutant, $ARF6Q_{67}L$—blocked Nef-mediated MHC-I downregulation in H9 cells

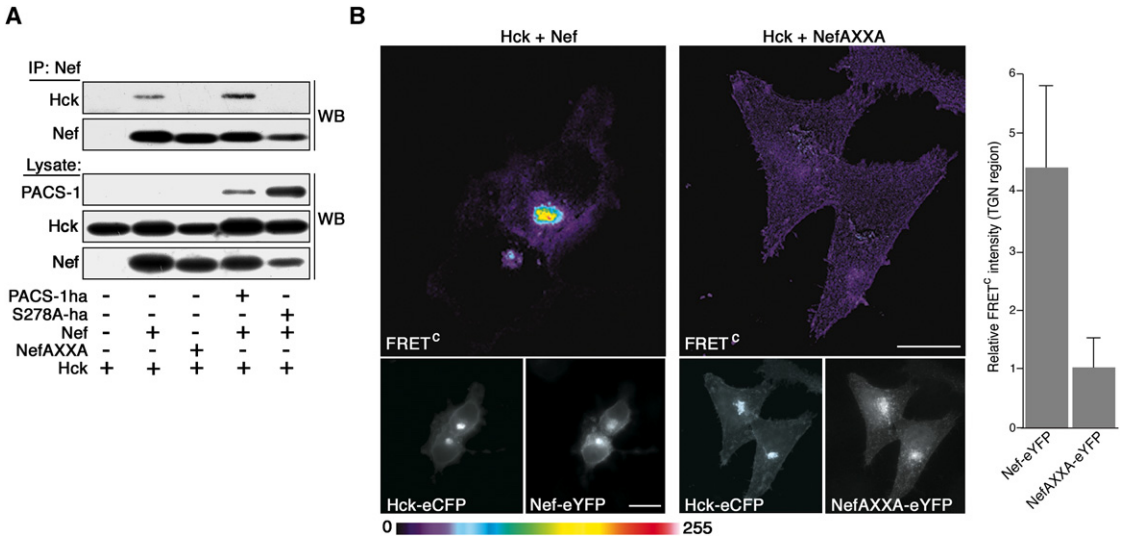

Figure 3. Nef $PXXP_{75}$ Recruits an SFK at the TGN

(A) A7 cells were infected with VV recombinants expressing the indicated proteins (total moi = 10, 16 hr) and were harvested, Nef constructs were immunoprecipitated from the membrane fractions with mAb M2, and coprecipitating Hck was detected by western blot. Bottom: Western blot showing expression of Hck, Nef, and the PACS-1 constructs.

(B) Left: Images of HeLa-$CD4^+$ cells coexpressing Hck-eCFP with Nef-eYFP or NefAxxA-eYFP were acquired using filters for CFP (lower left), YFP (lower right), and FRET (data not shown). $FRET^C$ (top) was calculated as described in the Experimental Procedures and is presented as a quantitative pseudocolor image with the corresponding pseudocolor scale (bottom). Scale bar, 20 μm. Right: Difference in $FRET^C$ between samples expressing Nef-YFP or NefAXXA-YFP. Error bars represent the mean ± SD of two independent experiments (n = 20).

(Figure S6). Thus, HIV-1 Nef requires a PI3K-stimulated, ARF6-controlled endocytic pathway to efficiently downregulate cell-surface MHC-I in $CD4^+$ T cells lines, and a functional PTEN is required to observe this effect.

Nef $EEEE_{65}$ and $PXXP_{75}$ Motifs Act Sequentially to Stimulate Nef-Associated PI3K Activity

The ability of Nef to bind the p85 regulatory subunit of PI3K (Linnemann et al., 2002), together with the requirement for Nef $EEEE_{65}$ and $PXXP_{75}$ to promote MHC-I downregulation by triggering the PIP_3-dependent activation of an ARF6-dependent endocytic pathway (Blagoveshchenskaya et al., 2002), raised the possibility that these two Nef motifs combine to stimulate a Nef-associated PI3K activity necessary to downregulate cell-surface MHC-I in $CD4^+$ T cells. To test this possibility, we first determined if Nef recruited PI3K in primary $CD4^+$ T cells. We expressed epitope (FLAG)-tagged Nef in IL-7-treated cells, then measured the amount of coprecipitating PI3K activity using an in vitro kinase assay (Figure 2A). In agreement with the immunofluorescence data (Figure 1E), the coprecipitating PI3K activity was blocked by LY294002, PIK-23, and PI-103, but not by PIK-112, demonstrating that Nef recruited a class I PI3K in vivo (Figure 2A). We extended these studies to H9 $CD4^+$ T cells and found that Nef mutants containing an $EEEE_{65} \rightarrow AAAA_{65}$ mutation (NefE4A), which disrupts binding of Nef to PACS-1, or a $PXXP_{75} \rightarrow AXXA_{75}$ mutation (NefAXXA), which blocks binding of Nef to SH3 domain-containing proteins, including SFKs, inhibited PI3K stimulation and MHC-I downregulation (Figures 2B and 2C). In agreement with the requirement for Nef $EEEE_{65}$ binding to PACS-1 to efficiently downregulate cell-surface MHC-I (Piguet et al., 2000), we found that the interfering mutant PACS-$1S_{278}A$, which inhibits binding of PACS-1 to Nef (Scott et al., 2003), reduced the amount of Nef-associated PI3K activity and inhibited MHC-I downregulation in H9 $CD4^+$ T cells (Figure 2D). However, as PI3K binds to the C-terminal region of Nef and not to the Nef $PXXP_{75}$ SH3 domain-binding motif (Linnemann et al., 2002), our results did not explain why NefAXXA failed to stimulate PI3K. Nef $PXXP_{75}$, but not $AXXA_{75}$, binds to SFKs, so we tested whether Nef-stimulated PI3K activity required an active SFK. Accordingly, we found that the SFK inhibitor PP2 blocked the stimulation of Nef-associated PI3K activity (Figure 2D; see also Figure 4A).

Nef $EEEE_{65}$-Mediated Targeting Enables $PXXP_{75}$ to Bind Src Family Kinases

Our results suggested that the Nef $EEEE_{65}$ and $PXXP_{75}$ motifs cooperate with a PP2-sensitive SFK to stimulate PI3K activity. Thus, Nef $EEEE_{65}$ may enable Nef $PXXP_{75}$ to bind an SFK, many of which localize to the TGN (Bard et al., 2002; Carreno et al., 2000). To test this possibility, we asked whether $EEEE_{65}$ regulates association of Nef with Hck (Figure 3A). We chose to examine Hck because Nef binding activates Hck (Lerner and Smithgall, 2002), and Hck is also required for rapid onset of HIV pathogenesis in transgenic mouse models (Hanna et al., 2001), while interfering fragments of Hck containing the SH3 domain block MHC-I downregulation (Chang et al., 2001). First, we coexpressed Hck with Nef or NefAXXA in cells

and, in agreement with an essential role for $PXXP_{75}$ to bind SFKs, we found that Nef, but not NefAXXA, coimmunoprecipitated Hck. Second, we coexpressed Nef and Hck with PACS-$1S_{278}$A or PACS-1, finding that PACS-$1S_{278}$A blocked the association of Nef with Hck on cell membranes, while PACS-1 had no effect (Figure 3A).

To determine whether HIV-1 Nef binds to SFKs at the TGN, we conducted an intermolecular FRET assay. We coexpressed Hck-CFP in HeLa-$CD4^+$ cells with Nef-YFP or NefAXXA-YFP, which localize to the TGN and emit a fluorescent signal with similar intensity (Figure 3B). To detect the intermolecular FRET signal, we exposed the cells to 436 nm light to excite Hck-CFP and measured fluorescence of Nef-YFP or NefAXXA-YFP at 535 nm. Only cells coexpressing Hck-CFP and Nef-YFP, but not NefAXXA-YFP, revealed a positive paranuclear FRET signal, indicating that the Nef-YFP binds to Hck-CFP at the TGN. Together, these results suggest that targeting to the TGN enables Nef to then bind an SFK, which subsequently stimulates PI3K activity required for Nef to downregulate cell-surface MHC-I.

Nef Binding to SFK Increases Association with PI3K

Our determination that PP2 inhibits Nef-associated PI3K activity and that Nef $PXXP_{75}$ binds to TGN-localized SFKs (Figures 2 and 3) suggested that bound SFKs enhance the recruitment of PI3K to Nef. In agreement with this possibility, we found that coexpression of Hck with Nef, but not NefAXXA, increased the amount of class I PI3K regulatory subunit p85 that coprecipitated with Nef and correspondingly increased the amount of Nef-associated PI3K activity (Figure 4A). Because Nef $PXXP_{75}$ binds and activates Hck (Lerner and Smithgall, 2002; and Figure S7), we asked whether the increased association of Nef with PI3K required SFK activity. We determined that, indeed, treatment of the cells with PP2 or coexpression of Nef with a catalytically inactive Hck mutant (Hck-KE; Lerner and Smithgall, 2002) blocked Hck activation (Figure S7) and the increase in coprecipitating PI3K activity, suggesting that an active SFK bound to $PXXP_{75}$ is required for Nef to stimulate recruitment of PI3K. The ability of Hck-KE to block Nef-mediated PI3K stimulation agrees with the report that a fragment of Hck containing the SH3 domain can block MHC-I downregulation in $CD4^+$ T cells (Chang et al., 2001). Next, we asked whether the association of PI3K with Hck was dependent upon Nef expression. We immunoprecipitated endogenous PI3K from cells that coexpressed Hck with Nef or NefAXXA and detected coimmunoprecipitating Hck by western blot (Figure 4B), finding that p85 coimmunoprecipitated Hck in the presence of Nef, but not NefAXXA. In addition to activating Hck, Nef also directly activates Src and Lyn (Trible et al., 2006). As Hck is most abundantly expressed in myeloid cells, we asked whether Src, which is broadly expressed, can similarly promote recruitment of PI3K to Nef. Accordingly, we found Src stimulated the amount of PI3K associated with Nef, but not NefAXXA. It also stimulated Nef-associated PI3K activity (Figure S8). Together, these findings suggest Nef serves as a scaffold to link activated SFKs to PI3K.

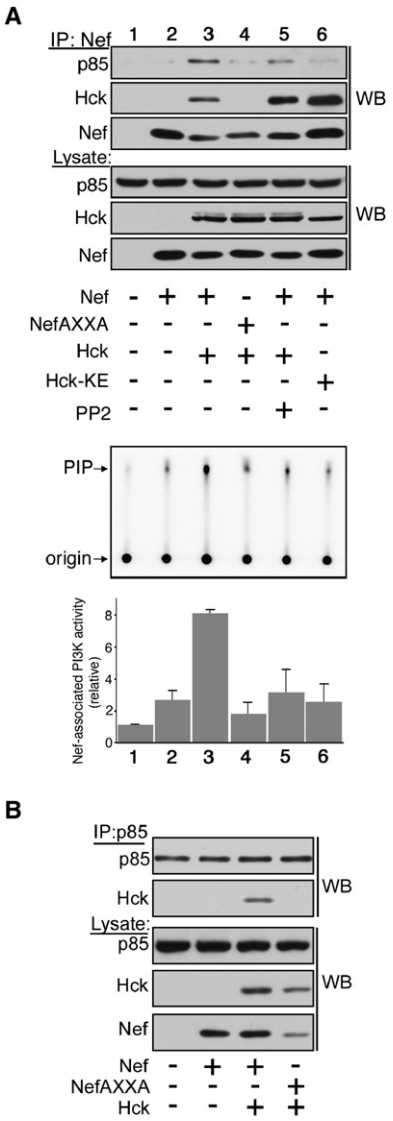

Figure 4. PI3K Recruitment by Nef Requires an Activated SFK Bound to Nef $PXXP_{75}$

(A) H9 $CD4^+$T cells infected with VV:WT or recombinant VV expressing the indicated proteins (moi = 6, 8 hr) were harvested, Nef proteins were immunoprecipitated, and coimmunoprecipitated Hck and p85 were detected by western blot. Coimmunoprecipitated PI3K activity was quantified as described in the legend to Figure 2. PP2 (10 μM) was added 2 hr prior to cell harvesting where indicated.

(B) H9 $CD4^+$ T cells infected with VV recombinants expressing the indicated proteins (moi = 6 total, 8 hr) were harvested, and p85 was immunoprecipitated from the extracts. Coimmunoprecipitated Hck and Nef were detected by western blot.

Bottom (A and B): Western blot showing expression of Nef and Hck and the amount of cellular p85.

Nef-SFK Recruits ZAP-70 to Stimulate PI3K

The requirement for an active SFK bound to Nef $PXXP_{75}$ to stimulate PI3K activity suggested that a Nef-bound SFK

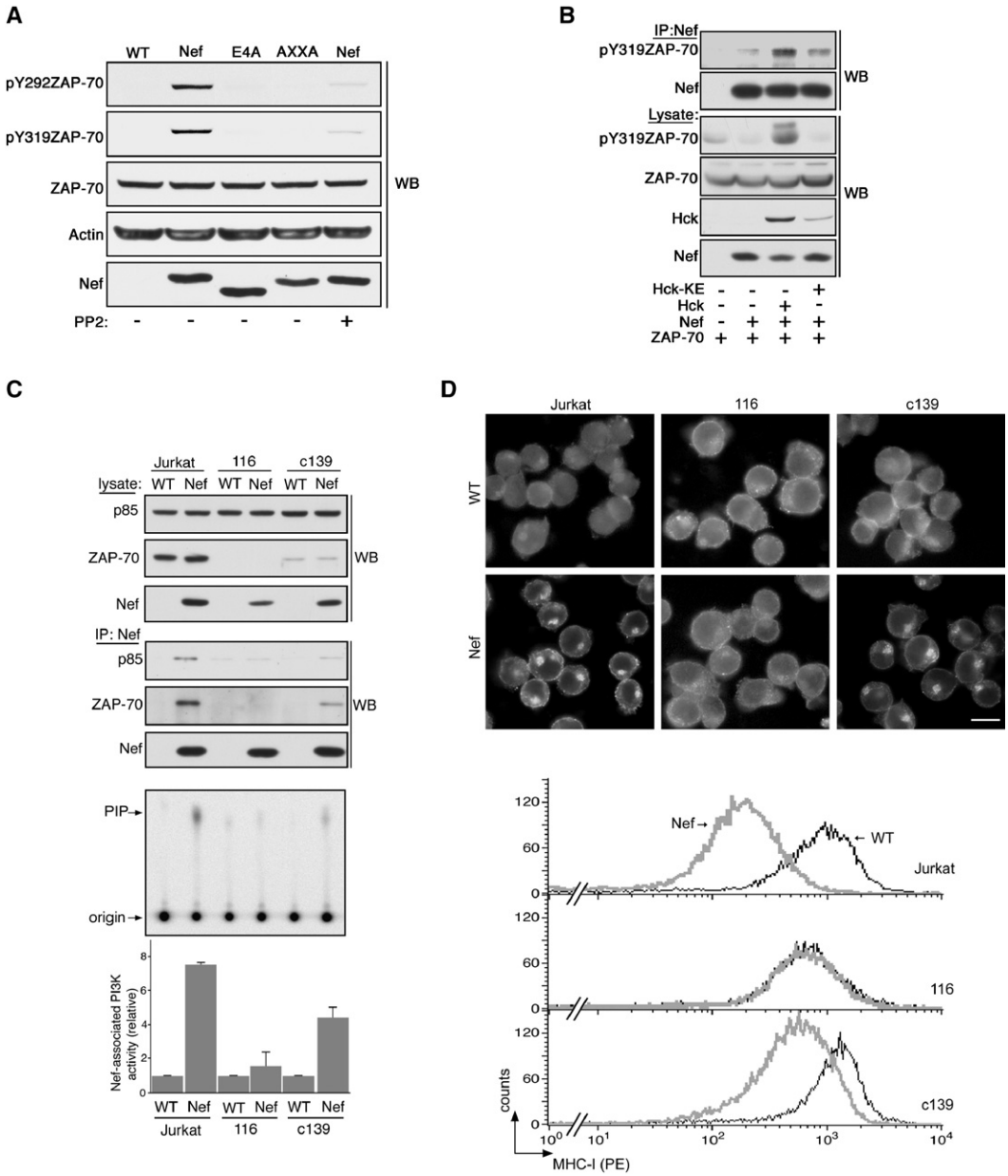

Figure 5. Nef/SFK Recruits and Activates ZAP-70 to Stimulate MHC-I Downregulation

(A) Jurkat CD4$^+$T cells infected with VV:WT or recombinant VV expressing the indicated proteins (moi = 10, 8 hr) in the absence or presence of PP2 (10 μM) were harvested, and pY_{292}ZAP-70 pY_{319}ZAP-70 were detected by western blot. Bottom: Western blot showing expression of the Nef constructs and the amount of cellular ZAP-70 and actin.

(B) 293T cells expressing ZAP-70 were infected with VV recombinants expressing the indicated proteins and were harvested, Nef proteins were immunoprecipitated, and coimmunoprecipitated phospho-ZAP-70 was detected by western blot. Bottom: Western blot showing expression of the Nef, Hck, and cellular ZAP-70.

(C) Replicate plates of Jurkat, JurkatP116, or JurkatP116.c139 cells were infected with VV:WT or VV:Nef/f (moi = 10, 8 hr). Nef was immunoprecipitated, and coimmunoprecipitating ZAP-70 and p85 were detected by western blot. The amount of coimmunoprecipitated PI3K activity was quantified as described in the legend to Figure 2. Top: Western blot showing the expression of Nef constructs and cellular ZAP-70 and p85.

(D) Jurkat, JurkatP116, or JurkatP116.c139 cells were infected with VV:WT or VV:Nef (moi = 10, 4 hr). The cells were fixed and stained with anti-MHC-I mAb W6/32 (scale bar, 10 μm). Bottom: Replicate cell cultures were stained with mAb W6/32 and processed for flow cytometry as described in the Experimental Procedures.

may directly phosphorylate PI3K. Yet we failed to detect phosphotyrosine on the p85 regulatory subunit that coimmunoprecipitates with Nef, suggesting that an activated SFK bound to Nef is necessary but not sufficient to stimulate PI3K. We thus sought to identify a substrate of the bound SFK that would stimulate PI3K. Recent studies

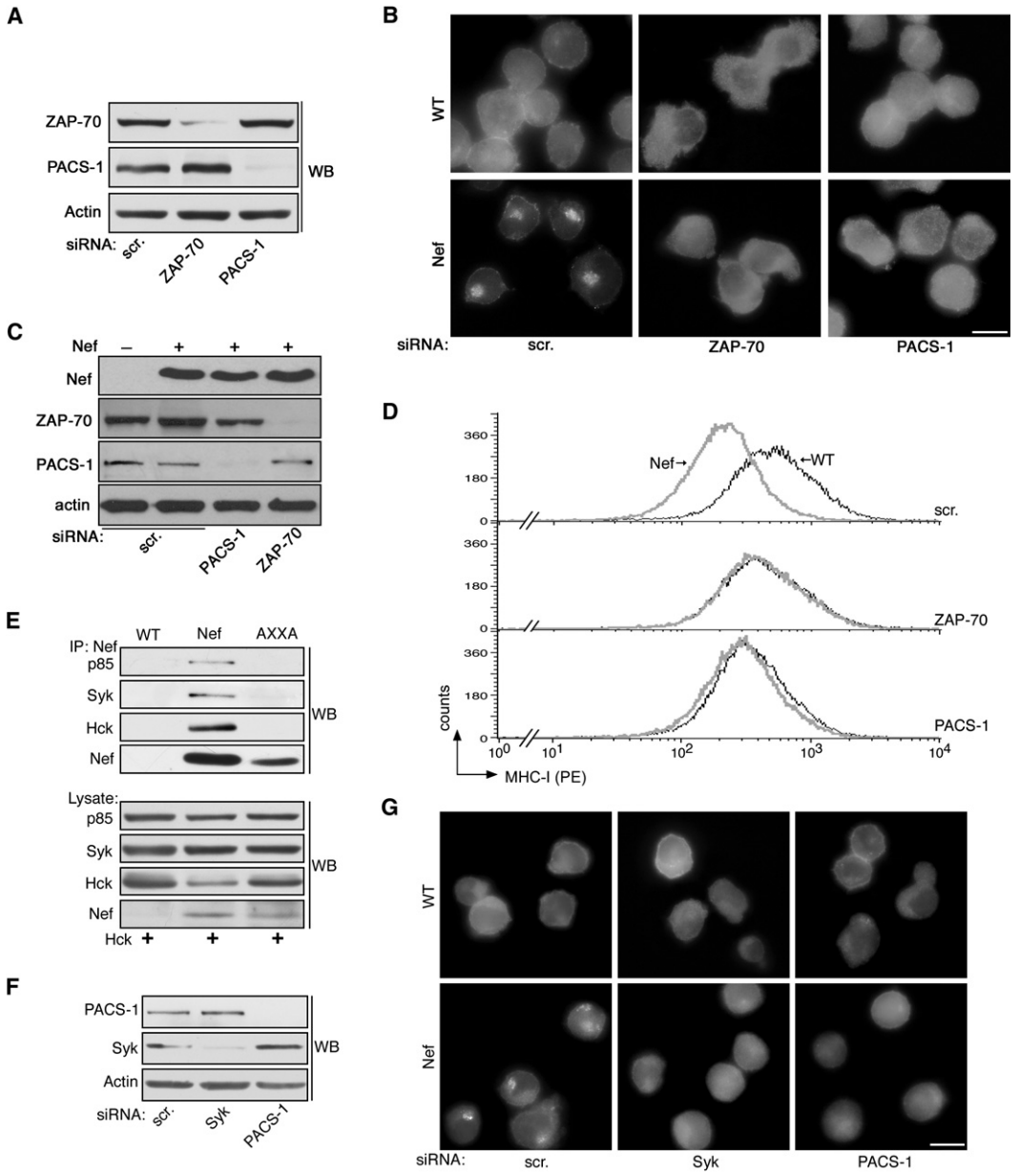

Figure 6. siRNA Depletion of PACS-1 and ZAP-70/Syk Block MHC-I Downregulation in H9 and Primary CD4$^+$ T Cells and in Promonocytic TF-1 Cells

(A) H9 CD4$^+$ T cells were nucleofected with pmaxGFP and either a control siRNA (scr) or siRNAs specific for ZAP-70 or PACS-1. After 60 hr, cells expressing GFP were collected by FACS, and the amounts of PACS-1, ZAP-70, and actin were determined by western blot.

(B) H9 CD4$^+$ T cells from (A) were infected with VV:WT or VV:Nef (moi = 10, 5 hr) and fixed, and MHC-I molecules were stained with mAb W6/32. Scale bar, 10 μm.

(C) Primary CD4$^+$ T cells isolated from a healthy donor were cultured in IL-7 and then nucleofected with a control siRNA (scr) or with siRNAs specific for ZAP-70 or PACS-1. After 60 hr, the cells were infected with VV:WT or VV:Nef (moi = 10, 16 hr) and analyzed by western blot.

(D) Primary CD4$^+$ T cells from (D) were analyzed by flow cytometry using mAb W6/32. Similar results were obtained using Nef$^+$ and Nef$^-$ pseudotyped viruses (data not shown).

(E) TF-1 cells infected with VV recombinants coexpressing Hck with Nef or NefAXXA (moi = 10 total, 8 hr) were harvested, and Flag-tagged Nef or NefAXXA were immunoprecipitated from the extracts. Coimmunoprecipitated Hck, Syk, and p85 were then detected by western blot. Bottom: Western blot showing expression of Nef, NefAXXA, and Hck constructs and the levels of cellular Syk and p85.

(F) TF-1 cells were nucleofected with pmaxGFP and either a control siRNA (scr) or siRNAs specific for Syk or PACS-1. After 48 hr, cells expressing GFP were collected by FACS and harvested, and the amounts of PACS-1, Syk, and actin were determined by western blot.

(G) TF-1 cells from (F) were infected with VV:WT or VV:Nef (moi = 10, 5 hr) and fixed, and MHC-I molecules were stained with mAb W6/32. Scale bar, 10 μm.

show that SFK phosphorylation of Syk or ZAP-70 forms a tyrosine motif that binds the C-terminal SH2 domain of p85 to stimulate PI3K- and ARF6-dependent phagocytosis (Moon et al., 2005; Zhang et al., 1998). We tested whether Nef/SFK usurped ZAP-70 to stimulate PI3K and found that Nef stimulated the tyrosine phosphorylation of ZAP-70 at Tyr_{292}, which is necessary for binding p85 (Figure 5A and Moon et al., 2005). By contrast, NefE4A, NefAXXA, or treatment of Nef-expressing cells with PP2 failed to activate ZAP-70, suggesting that $EEEE_{65}$ and binding of an active SFK to Nef $PXXP_{75}$ are essential for ZAP-70 activation. We also asked whether Hck could increase the association of Nef with ZAP-70, finding that Hck, but not inactive Hck-KE, increased the association of Nef with phosphorylated ZAP-70 (Figure 5B).

We then asked if ZAP-70 is required for Nef-mediated stimulation of PI3K activity and for Nef to downregulate cell-surface MHC-I. We expressed Nef in genetically paired Jurkat-derived cell lines that either lack Syk/ZAP-70 (P116 cells) or are rescued for ZAP-70 expression (P116.c39 cells, Figure 5C, top), immunoprecipitated the Nef molecules, and then quantified the amount of Nef-associated PI3K activity (Figure 5C). In agreement with our studies in H9 $CD4^+$ T cells, we found that Nef associated with PI3K in Jurkat cells. However, Nef failed to associate with PI3K in ZAP-70-deficient 116 cells. By contrast, Nef coimmunoprecipitated PI3K in the ZAP-70-rescued 139 cells. We used the ZAP-70-deficient and -rescued cell lines to determine whether ZAP-70 is required for Nef to downregulate cell-surface MHC-I and found that Nef failed to downregulate MHC-I in the ZAP-70-deficient 116 cells (Figure 5D). However, Nef downregulated cell-surface MHC-I in the ZAP-70-rescued 139 cells. As these experiments did not rely on PI3K inhibitors, results from these Jurkat cells were not confounded by the lack of PTEN.

siRNA Depletion of PACS-1 or ZAP-70 Inhibits Nef-Mediated MHC-I Downregulation in Primary $CD4^+$ T Cells

To establish that our results demonstrating the importance of ZAP-70 for the Nef-mediated downregulation of cell-surface MHC-I were not restricted to the Jurkat-derived cell clones, we asked whether siRNA depletion of ZAP-70 from H9 $CD4^+$ T cells would similarly block MHC-I downregulation. We treated H9 $CD4^+$ T cells with a control siRNA or with siRNAs that specifically depleted ZAP-70 or PACS-1 (Figure 6A), then expressed Nef in the siRNA-treated cells and measured MHC-I downregulation (Figure 6B). Depletion of ZAP-70 or PACS-1 repressed MHC-I downregulation, whereas the control siRNA had no effect. Next, to test whether Nef requires PACS-1 and ZAP-70 to downregulate MHC-I in primary $CD4^+$ T cells, we treated primary $CD4^+$ T cells with a control siRNA or with siRNAs that deplete PACS-1 or ZAP-70 (Figure 6C), then infected the cells with VV:WT or VV:Nef and measured downregulation of cell-surface MHC-I by flow cytometry (Figure 6D). Depletion of either PACS-1 or ZAP-70 inhibited Nef from efficiently downregulating MHC-I in primary $CD4^+$ T cells.

Finally, whereas ZAP-70 is expressed principally in T cells, its homolog Syk is broadly expressed in diverse cell types, including macrophages, which, like resting $CD4^+$ T cells, constitute a long-lived HIV-1 reservoir (Stevenson, 2003). Therefore, we asked whether Nef can use Syk in addition to ZAP-70 to stimulate PI3K. We determined that Nef, but not NefAXXA, associated with Hck, Syk, and PI3K in TF-1 promonocytic cells (Figure 6E). Accordingly, we treated TF-1 cells with siRNAs that depleted Syk or PACS-1 (Figure 6F). We then expressed Nef in the cells and found that siRNA depletion of Syk or PACS-1 blocked Nef-mediated downregulation of MHC-I (Figure 6G). Similar results were obtained using heterologous HeLa-$CD4^+$ cells, which also express Syk (Figure S9). Together, our results identify a novel Nef-SFK-ZAP-70/Syk-PI3K pathway that downregulates cell-surface MHC-I molecules in diverse HIV-1 target cells.

DISCUSSION

We identify a Nef-SFK-ZAP-70/Syk-PI3K signaling/trafficking pathway that HIV-1 Nef employs to downregulate cell-surface MHC-I. This pathway appears to be ubiquitous, since interference with SFKs, Syk/ZAP-70, or PI3K disrupts Nef-mediated MHC-I downregulation in all cell types examined that express a functional PIP_3 phosphatase, including primary $CD4^+$ T cells (Figures 1, 5, and 6 and Figures S3 and S9). Whereas SFK activation is usually triggered by C-terminal dephosphorylation, which relieves interdomain interactions that mask the active site, HIV-1 Nef overrides these inhibitory interactions by direct binding of its $PXXP_{75}$ motif to the SH3 domain on Hck, thereby unmasking the catalytic domain independent of dephosphorylation (Lerner and Smithgall, 2002). Recent studies show that this mode of Nef-mediated SFK activation also includes activation of Lyn and Src (Trible et al., 2006), supporting a role for Nef to directly activate SFKs expressed in multiple cell types. Interestingly, although Hck and Src have prominent roles at the plasma membrane, pools of these SFKs are present in other cellular compartments, including the Golgi/TGN (Bard et al., 2002; Carreno et al., 2000). Thus, Nef $EEEE_{65}$, which directs binding to PACS-1, enables Nef $PXXP_{75}$ to bind and directly activate SFKs sequestered in TGN-associated reservoirs. Our determination that PACS-$1S_{278}$A, PP2, and Hck-KE block the Nef-mediated stimulation of PI3K in multiple cell types, whereas expression of Hck or Src promotes recruitment of PI3K to Nef, supports this model (Figures 2, 4, and 5 and Figure S8).

Following activation, the Nef-associated SFK promotes recruitment and activation of ZAP-70, which is required to stimulate the PI3K-dependent downregulation of cell-surface MHC-I (Figures 5 and 6). In response to T cell receptor (TCR) ligation, ZAP-70 is recruited to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) present on the CD3ζ cytosolic domain, activating this Syk family kinase to phosphorylate

adaptor molecules that activate T cells (Deane and Fruman, 2004). We do not know if Nef directly binds ZAP-70. Nef lacks an ITAM motif, suggesting that ZAP-70 does not bind by this method. Interestingly, however, a mutant SIV Nef containing an ArgGln→TyrGlu substitution creates an ITAM motif that binds to and activates ZAP-70, mimicking the TCR and costimulatory signals that permit the aberrant and robust replication of the virus in unstimulated $CD4^+$ T cells, followed by rapid death of the host (Luo and Peterlin, 1997). The recent demonstration that SFK phosphorylation of ZAP-70 at the non-YXXM motif pY_{292} promotes binding of ZAP-70 to PI3K to increase receptor endocytosis suggests that HIV-1 usurps components of this cellular PI3K signaling pathway to stimulate MHC-I downregulation (Moon et al., 2005). Moreover, the ability of tyrosine phosphorylated Syk to also bind p85 (Moon et al., 2005) supports our determination that Nef can use Syk in place of ZAP-70 to stimulate PI3K and downregulate cell-surface MHC-I in cell types ranging from TF-1 promonocytic cells to HeLa-$CD4^+$ cells, which also express Syk (Figure 6 and Figure S9).

Our experiments identify the hierarchical role of the Nef $EEEE_{65}$ and $PXXP_{75}$ motifs in controlling MHC-I downregulation. Nef $EEEE_{65}$ and $PXXP_{75}$ combine to assemble a Nef-SFK-ZAP-70-PI3K complex that stimulates the ARF6-controlled endocytosis of cell-surface MHC-I, whereas Nef M_{20} promotes delivery of the endocytosed MHC-I to paranuclear compartments. The inability of Nef to block delivery of newly synthesized MHC-I to the surface of H9 $CD4^+$ T cells (Figure S4) supports our model that Nef triggers the downregulation of cell-surface MHC-I. The recent determination that Nef M_{20} is required for recruiting intracellular MHC-I to AP-1, which is necessary for endosome-to-TGN trafficking, is also consistent with our model (Roeth et al., 2004). However, our results differ from those of others (Kasper and Collins, 2003; Larsen et al., 2004), who reported that Nef-mediated MHC-I downregulation was controlled by a PI3K-independent mechanism and that Nef blocks delivery of newly synthesized MHC-I to the cell surface. Several factors may have contributed to these differing results. First, these authors relied on PTEN-deficient cells to ascertain the role of PI3K in Nef-mediated downregulation of MHC-I, which possess inordinately high levels of PIP_3 and thus respond poorly to PI3K inhibitors (Deane and Fruman, 2004). The ability of PTEN to rescue sensitivity of Nef-expressing U373 cells to LY294002 confirms the shortcoming of these cell lines to study Nef's role in HIV-1 immunoevasion (Figure 1) and underscores the large number of conflicting studies in T cell biology attributed to the use of PTEN-deficient cell lines (Astoul et al., 2001). Second, that cell-surface MHC-I is endocytosed by an ARF6-dependent pathway and is not blocked by dynamin/AP-2 mutants (Blagoveshchenskaya et al., 2002; Le Gall et al., 2000) may also have confounded elucidation of this MHC-I downregulation pathway (Swann et al., 2001). Third, in contrast to others (Williams et al., 2005), we found that the inability of NefE4A and NefAXXA mutants to downregulate MHC-I was due, at least in part, to specific defects in the PI3K signaling pathway. The various studies of Nef expression may also define two physiologically relevant modes of MHC-I downregulation—a signaling-dependent mode identified here, which triggers downregulation of cell-surface MHC-I, and a stoichiometric mode, which blocks transport of newly synthesized MHC-I molecules to the cell surface (Kasper and Collins, 2003; Roeth et al., 2004). Whether the two modes of MHC-I downregulation reflect reservoir- or host cell-activation-state-dependent activities of Nef warrants further investigation.

The recent identification of isoform-specific PI3K inhibitors provides new strategies to combat disease. For example, PI-103 constrains the growth of gliomas in vivo (Fan et al., 2006). Thus, the ability of PI-103 to suppress Nef-mediated downregulation of MHC-I in $CD4^+$ T cells (Figure 1) suggests that HIV-1 may be an additional target for this novel inhibitor. Importantly, Nef induces macrophages to release chemokines, luring nonactivated T cells and causing them to become permissive to the virus, thereby creating a very long-lived HIV-1 reservoir unlikely to be eradicated by HAART (Stevenson, 2003). Thus, the ability of PI-103 to inhibit Nef-mediated MHC-I downregulation in naive IL-7-treated primary $CD4^+$ T cells suggests a novel strategy to combat the virus (Figure 1). Interestingly, whereas PI-103 is a multitargeted PI3K inhibitor, quinazolinone purines, including PIK-23, are exquisitely selective for p110δ, which is largely restricted to leukocytes. As mice lacking an active p110δ are viable (Okkenhaug et al., 2002), specifically targeting this PI3K isoform may provide a novel approach to disrupt HIV-1 in leukocyte reservoirs without affecting PI3K activity in other tissues.

The Nef-SFK-ZAP-70/Syk-PI3K axis that we report here may have implications beyond solely controlling MHC-I downregulation. The same Nef motifs controlling MHC-I downregulation are also involved in PAK2 activation, in the blocking of T cell maturation, and perhaps in MHC II-mediated antigen presentation. The Nef $EEEE_{65}$ and $PXXP_{75}$ SH3 motifs and PI3K are all required to activate PAK2, which further arms the HIV-1 antiviral counterattack (Das and Jameel, 2005 and our unpublished data). Similarly, Nef requires the Nef $EEEE_{65}$ and $PXXP_{75}$ motifs to disrupt maturation of $CD34^+$ thymocytes (Stove et al., 2003), and PI3K inhibitors block the Nef-induced increase in cell-surface levels of immature MHC-II and FasL, raising the possibility that Nef turns the host's own apoptotic arsenal against itself, such that infected cells kill the very same $CD8^+$ CTLs that target them by Fas-FasL binding (Das and Jameel, 2005; Peterlin and Trono, 2003; Zauli et al., 1999). Together, our results explain the role of the Nef $EEEE_{65}$ and $PXXP_{75}$ motifs for mediating HIV-1 immunoevasion and potentially additional facets of HIV-1 disease by directing the formation of a Nef-SFK-ZAP-70/Syk-PI3K multikinase signaling complex in diverse cell types, and in doing so, we suggest new strategies to block this pathway in the treatment of AIDS.

EXPERIMENTAL PROCEDURES

Cells and Recombinant Virus

293T, HeLa-CD4$^+$, A7 melanoma, U373 astrocytoma, and Jurkat CD4$^+$ leukemic T cells were cultured as described (Blagoveshchenskaya et al., 2002; Crump et al., 2003). Jurkat-derived P116 and P116.c139 cells (provided by A. Weiss) and H9 CD4$^+$ T cells were cultured in RPMI-1640 supplemented with 10% FBS. TF-1 promonocytic cells were cultured in RPMI-1640 supplemented with 10% FBS and 2 ng/ml GM-CSF (Sigma).

Naive CD4$^+$ T cells were isolated from freshly drawn blood or from leukapheresed cells donated by four healthy volunteers using a MACS CD4$^+$ T cell isolation kit (Miltenyi Biotec). The purity of the CD4$^+$ T cell population was verified by FACS using PE-conjugated anti-CD4 (mAb Leu-3a, BD). Isolated cells were cultured in RPMI 1640 containing 10% FBS and supplemented with 2 ng/ml IL-7 for 4 days or treated with IL-2 (1 U/ml for siRNA studies or 5 U/ml for PI3K studies; Sigma) and 1 μg/ml PHA (Sigma) prior to infection.

HIV-1 NL4-3 was grown and titered as described (Scholz et al., 2005). Vaccinia virus (VV) expressing flag-tagged Nef, NefE4A, NefAXXA, and NefAXXA-PI3K*, as well as PI3K*, ARF6, ARF6Q$_{67}$L, ARNO, ARNOE$_{156}$K, Src, PACS-1, and PACS-1S$_{278}$A were generated and titered on BSC-40 cells as described (Blagoveshchenskaya et al., 2002; Ely et al., 1994; Scott et al., 2003). VV expressing Hck or Hck-KE cDNAs (provided by T. Smithgall) or ZAP-70 cDNA (provided by A. Weiss) were prepared as described (Blagoveshchenskaya et al., 2002). To produce the HIV pseudotyped viruses NL4-3ΔG/P-EGFP and NL4-3ΔG/P-EGFP/Δ Nef, 293T cells were cotransfected with the packaging vector pCMVΔR8.2, pMD.G, which expresses VSV-G and either pNL4-3ΔG/P-EGFP or pNL4-3:ΔG/P-eGFP/ΔNef (provided by P. Klotman). At 48 hr postinfection, viruses were collected and titered on 293T cells based on GFP expression. Cells were infected with NL4-3ΔG/P-EGFP or NL4-3:ΔG/P-eGFP/ΔNef (moi = 5) in the presence of 6 μg/ml polybrene (Sigma).

PI3K Inhibitors and siRNA

PI-103, PIK-23, and PIK-112 (Knight et al., 2006), and LY294002 (Calbiochem), were used as indicated. For cell experiments, inhibitors were added at 21–48 hr postinfection; cells were processed for flow cytometry at 24–60 hr postinfection, depending upon the donor. Control (scr) siRNA and siRNAs specific for PACS-1, ZAP-70, Syk, and PTEN (Dharmacon) were nucleofected into cells according to the vendor's instructions (Amaxa). In some experiments, cells were conucleofected with pmaxGFP to enrich transfected cell populations by FACS.

FACS and Flow Cytometric Analysis

Where indicated, GFP$^+$ cells were selected using a FACS Vantage flow cytometer cell sorter. For flow cytometry, cells were washed and resuspended in FACS buffer (PBS [pH 7.2] containing 0.5% BSA and 0.1% NaN_3). Cells were incubated with mAb W6/32 (1:4000) or mAb BB7.1 (anti-HLA-A2.1, 1:400; BD) at 4°C for 1 hr. An isotype-matched antibody was used as a negative control. Cells were then washed and incubated with PE-conjugated donkey anti-mouse IgG (1:400; Jackson IR) at 4°C for 30 min. Cells were washed and analyzed by listmode acquisition on a FACSCalibur (BD) using CellQuest acquisition/analysis software (BD).

FRET Analysis

Nef-YFP and NefAXXA-YFP were constructed by subcloning the Nef and NefAXXA cDNAs into peYPF-N1 (Clontech); Hck-eCFP was constructed by subcloning the Hck cDNA into peCFP-N1 (Clontech). HeLa-CD4$^+$ cells were cotransfected with pHck-eCFP and either pNef-eYFP or pNefAXXA-eYFP (Fugene, Roche). After 24 hr, images were captured using a 63× oil immersion objective lens and a cooled CCD camera and recorded using MetaMorph software (Molecular Dynamics). To measure FRET, three images were acquired in the beginning with a CFP filter set (λ_{ex} 436/10 nm, λ_{em} 470/30 nm), followed by a YFP filter set (λ_{ex} 500/20 nm, λ_{em} 535/30 nm) and then a FRET filter set (λ_{ex} 436/10, λ_{em} 535/30 nm). Images were background subtracted, and the corrected FRET (FRET^C) was obtained from the raw FRET images by subtracting the bleedthrough signals emitted through the CFP and YFP channels from cells expressing Hck-CFP, Nef-YFP, or NefAXXA-YFP alone on a pixel-by-pixel basis. The percentage of bleedthrough from the eCFP and eYFP fluorescent signals (typically 45% CFP and 25% YFP) was determined by dividing the average intensity of the image obtained using the FRET filter configuration by the average intensity of the image obtained using the CFP or YFP filter configuration, respectively. The difference in FRET^C between cells expressing Nef-YFP or NefAXXA-YFP was quantified by the following formula: $[(\text{mean pixel intensity})_T \bullet \text{area}] - [(\text{mean pixel intensity})_B \bullet \text{area}]/[(\text{mean pixel intensity})_C \bullet \text{area}] - [(\text{mean pixel intensity})_B \bullet \text{area}]$, where T = TGN, C = cytosol, and B = background in the extracellular space.

Coimmunoprecipitation, Western Blot, and Antibody Uptake

Cells infected with the indicated VV recombinants were harvested in PBS containing 1% NP40, protease inhibitors (0.5 mM PMSF and 0.1 μM each of aprotinin, E-64, and leupeptin) and phosphatase inhibitors (1 mM Na_3VO_4 and 20 mM NaF). Where indicated, cells were treated with 10 μM PP2 (Calbiochem) or 5 μM LY294002 prior to harvesting. In some experiments, cells were harvested in PBS and inhibitor cocktails without detergent, and then 100,000 × g membrane pellets were collected and resuspended in PBS containing 1% TX-100 plus inhibitors. FLAG-tagged Nef constructs were immunoprecipitated with mAb M2-agarose (Sigma), and coimmunoprecipitating proteins were detected by western blot. The following antibodies were obtained as indicated: mAb HA.11 (Covance); anti-Hck, anti-Syk, and anti-SHIP-1 (Santa Cruz); anti-Src, anti-p85, anti-ZAP-70, and anti-SHIP-2 (Upstate); anti-pY$_{418}$ (Biosource); anti-phospho$_{292}$ZAP-70 (BD); anti-phospho$_{319}$ZAP-70 and anti-PTEN (Cell Signaling); anti-actin (Chemicon); anti-Nef (AIDS Research and Reference Reagent Program, NIH); and anti-PACS-1(703) (Scott et al., 2006). Antibody uptake using mAb W6/32 was performed as described (Blagoveshchenskaya et al., 2002). Residual cell-surface-bound antibody was removed by a brief acid strip (1% acetic acid, 0.5 M NaCl [pH 3.0]; 30 s) prior to fixation.

PI3 Kinase Assay

Immunoprecipitates from H9 cells expressing the designated Nef constructs were resuspended in assay buffer (20 mM HEPES [pH 7.4], 30 mM $MgCl_2$, and 20 μM ATP) and then incubated with 0.2 mg/ml PI (Sigma) and 10 μCi [γ-^{32}P]ATP for 15 min at RT. Phospholipids were extracted with an HCl, chloroform/methanol (1:1) solution, spotted on TLC plates (Fisher), and separated in a solvent mixture composed of chloroform/methanol/water/NH_4OH (45:35:8.5:1.5). ^{32}P-PIP was visualized by autoradiography and quantified by phosphorimage analysis.

Immunofluorescence Microscopy

Cells were infected with the designated VV recombinants and either fixed or pelleted (suspension cell lines) onto poly-L-lysine-treated coverslips and then fixed with 4% paraformaldehyde and processed for immunofluorescence. Images were captured using a 63× oil immersion objective on a Leica DM-RB microscope and Hamamatsu C4742-95 digital camera and processed with Scion Image 1.62.

Supplemental Data

The Supplemental Data include nine supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/1/2/121/DC1/.

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