Induction of Fas Ligand Expression by HIV Involves the Interaction of Nef with the T Cell Receptor ζ Chain

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Summary

During HIV/SIV infection, there is widespread programmed cell death in infected and, perhaps more importantly, uninfected cells. Much of this apoptosis is mediated by Fas-Fas ligand (FasL) interactions. Previously we demonstrated in macaques that induction of FasL expression and apoptotic cell death of both CD4+ and CD8+ T cells by SIV is dependent on a functional nef gene. However, the molecular mechanism whereby HIV-1 induces the expression of FasL remained poorly understood. Here we report a direct association of HIV-1 Nef with the ζ chain of the T cell receptor (TCR) complex and the requirement of both proteins for HIV-mediated upregulation of FasL. Expression of FasL through Nef depended upon the integrity of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR ζ chain. Conformation for the importance of ζ for Nef-mediated signaling in T cells came from an independent finding. A single ITAM motif of ζ but not CD3ε was both required and sufficient to promote activation and binding of the Nef-associated kinase (NAK/p62). Our data imply that Nef can form a signaling complex with the TCR, which bypasses the requirement of antigen to initiate T cell activation and subsequently upregulation of FasL expression. Thus, our study may provide critical insights into the molecular mechanism whereby the HIV-1 accessory protein Nef contributes to the pathogenesis of HIV.

Key words: Jurkat • immunoreceptor tyrosine-based activation motif • Nef-associated kinase • activation-induced cell death • apoptosis

In HIV/simian immunodeficiency virus (SIV) infection, the nef gene plays a key role in viral replication and progression of disease. This is based on studies in macaques and humans, who remain asymptomatic or long-term nonprogressing when infected with an SIV mutant lacking a nef gene or HIV with multiple nef deletions, respectively (1–3). More recently, a study using a transgenic mouse model has demonstrated that Nef harbors a major determinant for HIV-induced pathogenicity (4). Despite the considerable importance of Nef for HIV/SIV pathogenesis, its function at the molecular level is poorly understood. At least three

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Abbreviations used in this paper: aa, amino acid(s); AICD, activation-induced cell death; ITAM, immunoreceptor tyrosine-based activation motif; mu, mutant; NAK, nef-associated kinase; RT, reverse transcriptase; SIV, simian immunodeficiency virus.
lecular mechanism integrating this observation into other documented effects of Nef is lacking.

The concept of Nef interfering with early events emanating from the TCR could explain its dual effects on T cell activation and FasL expression, since both functions are regulated by the TCR complex (23–25). Here we report that HIV-mediated upregulation of FasL in T cells is dependent on the association of Nef with the TCR ζ chain. By demonstrating that Nef directly targets the TCR of the infected cell, we provide novel insight into the molecular function of Nef in HIV infection.

Materials and Methods

Cell Lines and Antibodies. Generation of Jurkat cell lines constitutively expressing CD8 tag or CD8-Nef chimeras was described recently (8, 26). Jurkat, J.CaM.1 (Lck constitutively expressing CD8 tag), or CD8-Nef chimeras was described previously (8, 26). The mAbs against the AU-5 (anti-CD16/anti-CD8 Beads. The mAbs against the AU-5/ AU-1 epitope and FasL (NOK-1) were purchased from His Diagnostics and Pharmingen.

Plasmid Constructions. Generation of the CD8-Nef (SF2) and CD16/ζ chimeras as well as the COOH-terminal-tagged Nef (AU-1) was described previously (8, 26, 28). Fusion proteins between CD16 and individual ζ ITAMs (ITAM 1, amino acids [aa] 1–70; ITAM 2, aa 70–110; ITAM 3, aa 110–141) were generated as described previously (28). The mutations in CD16ζ as well as in Nef/CN.94 were generated by a two-step PCR procedure and cloned into the pRcCMV vector (Invitrogen). In CD16ζ, the tyrosine residues in three ITAM motifs (two tyrosine residues in each ITAM) were mutated to alanines. In Nef/CN.94, the ζ ITAM motifs (ITAM 1, amino acids [aa] 1–70; ITAM 2, aa 70–110; ITAM 3, aa 110–141) were generated as described previously (28). The mutations in CD16ζ were expressed in tumor cells (26). In CD16ζ-mu, the tyrosine residues in three ITAM motifs (two tyrosine residues in each ITAM) were mutated to alanines. In Nef/CN.94, the ζ ITAM motifs (ITAM 1, amino acids [aa] 1–70; ITAM 2, aa 70–110; ITAM 3, aa 110–141) were generated as described previously (28). The mutations in CD16ζ were expressed in tumor cells (26). For the generation of recombinant baculoviruses, the “bac to bac” system was used (Bio-Rad Laboratories).

Protein Expression Assays. Transfections into 293T cells, metabolic labeling with [35S]-Translabel, immunoprecipitation, Western blot, and in vitro kinase assays were performed as described previously (8, 26). The immunoprecipitates were washed three times (wash buffer: 1% NP-40, 450 mM NaCl, 50 mM Tris-HCl [pH 8], 1 mM EDTA). To show an interaction between Nef and ζ, extraction and washing buffers contained 1% Brij instead of 1% NP-40. FasL promoter activity was tested as described previously (29) by cotransfection of pFasL-Luc (provided by Xiangdong Liu, University of California, San Francisco, CA [27]). J.RT3-T3.5 cells express CD16 and individual ζ ITAMs (ITAM 1, amino acids [aa] 1–70; ITAM 2, aa 70–110; ITAM 3, aa 110–141) were generated as described previously (28). The mutations in CD16ζ as well as in Nef/CN.94 were generated by a two-step PCR procedure and cloned into the pRcCMV expression vector (Invitrogen). In CD16ζ-mu, the tyrosine residues in three ITAM motifs (two tyrosine residues in each ITAM) were mutated to alanines. In Nef/CN.94, the ζ ITAM motifs (ITAM 1, amino acids [aa] 1–70; ITAM 2, aa 70–110; ITAM 3, aa 110–141) were generated as described previously (28). The mutations in CD16ζ were expressed in tumor cells (26). For the generation of recombinant baculoviruses, the “bac to bac” system was used (Bio-Rad Laboratories).

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In Vitro HIV Infection of Jurkat or Jurkat Mutant Cell Lines. Cells (5 × 10⁴) were superinfected with 1 ml of HIV IIIB (1.6 × 10⁶ cpm/ml, reverse transcriptase [RT] activity 1000 mU/ml) in the presence of a helper virus (29). The supernatant was collected for 48 h. Cell culture supernatants were collected on day 5 for analysis of p24 by ELISA or RT activity by Quan-T-R kit (Amersham Pharmacia Biotech).

A analysis of FasL expression by Flow Cytometry and Immunoprecipitation. To assess cell surface FasL expression on HIV-infected cells or transiently transfected Jurkat TAg cells, the metaproteinase inhibitor BB2116 (British Biotech [30]) was added to the medium 4–6 h before the assay to enhance cell surface FasL expression. In brief, cells were stained with 20 μl of biotin-conjugated anti-human FasL mAb (NOK-1; Pharmingen) followed by 5 μl of PE-conjugated streptavidin (Sigma). Labeled cells were analyzed on a FACScan® (Becton Dickinson). Isotype-specific mAbs of irrelevant specificity were used as negative controls (Dako Diagnostics). To assess expression of whole FasL protein, 35S-labeled cells (5 × 10⁶) were immunoprecipitated for FasL using anti-FasL-specific mAb (NOK-1) as described previously (30).

Results

Requirement of the TCR ζ Chain for Binding of Nef-associated Kinase (p62) to Nef. As shown previously, Nef associates with a serine kinase, termed p62 or Nef-associated kinase (NAK [31]). The Nef-NAK interaction is complex: Nef stimulates the phosphorylation/activation of NAK, and it is only in this activated form that NAK can bind Nef (32). This suggests that Nef must act upstream of NAK to promote NAK activation. Our previous results showing that Nef interfered with early signals emanating from the TCR suggested it may interact with a component of the TCR signaling complex. This prompted us to study Nef-mediated NAK/p62 activation in cell lines with TCR signaling defects. CD8-Nef chimeras (CD8-Nef), containing the extracellular domain of CD8α fused to Nef, were stably transfected into wild-type Jurkat and a variety of Jurkat mutant cell lines lacking either Lck (I.CaM.1), CD45 (CD45ζ), or the entire TCR signaling complex (R.T3-T3.5). Expression of CD8-Nef in these cell lines was verified by metabolic labeling and immunoprecipitation (Fig. 1 B). The Nef chimeras from these transfected lines were immunoprecipitated and subjected to an in vitro kinase assay. NAK/p62 association was observed in all cell lines except the TCR−cells (Fig. 1 A). The latter result was confirmed in a second, independently transfected cell clone (data not shown).

Next we asked whether NAK binding could be restored in cells lacking the TCR complex by stable transfection with TCR-ζ or CD3ε fused to the extracellular domain of CD16 (CD16ζ and CD16ε). These TCR subcomponents contain signaling motifs (immunoreceptor tyrosine-based T cell activation motifs [ITAMs]), which are required and sufficient for T cell activation (28, 33). After obtaining single cell clones, expression of the chimeras was verified by metabolic protein labeling and FACS® analysis (data not shown). In several attempts, we were unable to coexpress CD8-Nef (CN) with CD16ε in TCR−cells. Cell clones that were obtained either showed no detectable CN or CD16 expression or died rapidly. The effect resembled activation-induced cell death (AICD) by Nef as reported previously (8). Coexpression of CN and CD16ε was achieved; however, the obtained cell clones had a low CN as well as additional 48 h. Cell culture supernatants were collected on day 5 for analysis of p24 by ELISA or RT activity by Quan-T-R kit (Amersham Pharmacia Biotech).
as CD16 surface expression (Fig. 1 D, lane 3). Therefore, we constructed ζ chimeras containing the three individual ζ ITAMs in isolation (CD16ζ1, 2, or 3; see Materials and Methods for details). In a separate construct, the tyrosine residues in all three YXXL motifs of CD16ζ were mutated to alanines (CD16ζmu). We failed to coexpress the first ζ ITAM with Nef. However, NAK/p62 binding to Nef was reestablished in the TCR− cells by coexpression with the second or third ITAM of ζ (Fig. 1 C, lanes 4 and 5). In these latter cell lines, expression of CD16ζ1 and 2 as well as CD8-Nef decreased significantly over time (data not shown), indicating that coexpression of both proteins was not favorable. The difficulties regarding the coexpression of the individual ζ ITAMs with CD8-Nef may be explained by studies published by Combadiere et al. (34) showing that in particular the first ζ ITAM but much less the second and third are capable of inducing apoptosis when activated. The signaling-defective ζ chain (CD16ζμu) expressed well, but NAK/p62 binding to Nef was greatly reduced (lane 6). No NAK/p62 binding was observed by coexpression of CD3ζ (lane 3). Since NAK binding to Nef was not completely negative with CD16ζμu, the Nef–ζ complex may recruit additional signaling molecules to the plasma membrane which are important for NAK activation. Assuming that the effects of the first ζ ITAM would be similar to ITAM 2 and 3, it appeared that at least one functional ITAM of the CD3 ζ chain was required for binding of p62/NAK to Nef.

The functional link between Nef, ζ, and NAK was confirmed by transient transfection assays in a heterologous system. As shown in Fig. 1 E, cotransfection of CD16ζ (lanes 4 and 5) but not CD16ζ (lanes 2 and 3) significantly increased binding of p62/NAK to Nef. A minimal increase was seen after cotransfection of CD16ζμu (lanes 6 and 7), which paralleled the small effect seen in Fig. 1 C, lane 6. Thus, no other T cell–specific components except the functional ITAM(s) from the TCR ζ chain, were required for NAK activation and NAK/Nef association in 293T cells.
proteins was obtained by coimmunoprecipitation after transient transfection into COS cells and subsequent in vitro kinase assay (data not presented).

Upregulation of FasL expression by HIV requires both intact Nef and TCR-ζ chain. We have previously shown that the upregulation of FasL in SIV infection requires an intact nef gene (22). In general, the level of cell surface FasL expression is quite low when analyzed by FACST® even when metalloproteinase inhibitors are used which block cleavage of FasL from the cell surface. In view of this difficulty, we used additional experimental approaches to analyze Nef-mediated FasL expression (see below). Since stimulation of TCR-ζ effectively upregulates FasL expression (34, 36), we speculated that the interaction of Nef with TCR-ζ would lead to a similar effect. First, to show that HIV-Nef is required for FasL upregulation, we infected Jurkat with wild-type HIV (NL4-3SF2Nef) or a mutant lacking the nef gene (NL4-3ΔNef) (Fig. 3). Little if any FasL is seen on cells infected with Nef-deleted HIV, thus confirming our previous results with SIV. The level of viral replication in Jurkat cells was comparable, as determined by RT activity (NL4-3SF2Nef, 4.6 × 10^3 cpm; NL4-3ΔNef, 5.8 × 10^3 cpm). Uproregulation of FasL by HIV is also lost in mutant Jurkat cells lacking the TCR complex, whereas cells reconstituted with the TCR-ζ chain. (A) Anti-Nef (CD8) immunoprecipitation, then anti-ζ Western blot (WB). (B) Anti-ζ immunoprecipitation, then anti-Nef Western blot from wild-type Jurkat cells stably transfected with CD8 tag (Cont.), and CD8-Nef chimeras containing full-length Nef expressed in the cytoplasm (CD8-Nef.cyt.), the NH2-terminal 49 amino acids of Nef (CD8-Nef.49), the NH2-terminal 94 amino acids (CD8-Nef.94), and CD8-Nef.94PXmu in which the PxxP motif has been mutated. (C) Control immunoprecipitation of CD8-Nef chimeras from 35S-labeled cells to show a comparable protein expression (*). (D) Nef-ζ association after baculovirus coinfection of Hi5 cells. Control anti-Nef Western blot (WB) after anti-Nef (AU-1) immunoprecipitation from Hi5 cells infected with wild-type Nef or Nef with a mutated PxxP motif.

Next, FasL upregulation was studied in Jurkat and TCR-ζ mutant cell lines using a FasL promoter/luciferase reporter construct. The latter has been shown to be stimulated in transient assays by the HTLV I Tax protein (29). Nef stimulated the FasL promoter in Jurkat and TCR-ζ mutant cells reconstituted with the TCR-ζ chain.

Figure 2. Association of membrane-associated Nef with the TCR-ζ chain. (A) Anti-Nef (CD8) immunoprecipitation, then anti-ζ Western blot (WB). (B) Anti-ζ immunoprecipitation, then anti-Nef Western blot from wild-type Jurkat cells stably transfected with CD8 tag (Cont.), and CD8-Nef chimeras containing full-length Nef expressed in the cytoplasm (CD8-Nef.cyt.), the NH2-terminal 49 amino acids of Nef (CD8-Nef.49), the NH2-terminal 94 amino acids (CD8-Nef.94), and CD8-Nef.94PXmu in which the PxxP motif has been mutated. (C) Control immunoprecipitation of CD8-Nef chimeras from 35S-labeled cells to show a comparable protein expression (*). (D) Nef-ζ association after baculovirus coinfection of Hi5 cells. Control anti-Nef Western blot (WB) after anti-Nef (AU-1) immunoprecipitation from Hi5 cells infected with wild-type Nef or Nef with a mutated PxxP motif.

Figure 3. Nef is required for FasL upregulation. Jurkat cells were infected with (A) wild-type HIV-1 (NL4-3SF2Nef) or (B) SF2 lacking the nef gene (NL4-3ΔNef). After 48 h, FasL expression (solid line) was assessed by flow cytometry and compared with staining with a control mAb (dashed line). The level of viral replication in Jurkat cells was comparable as determined by RT activity (NL4-3SF2Nef, 4.6 × 10^3 cpm; NL4-3ΔNef, 5.8 × 10^3 cpm).
chain. No effect was seen using the Nef.PXmu construct or the TCR \( \beta \) Jurkat cell line (Fig. 6). These assays confirmed that a functional Nef protein and the TCR \( \beta \) chain were both required and sufficient to upregulate FasL in T cells.

Discussion

In general, the interaction between Fas and FasL plays an important role in the homeostatic regulation of normal immune responses (37). Stimulation of the TCR-CD3 complex in T cells causes upregulation of FasL and eventually leads to AICD or apoptosis (23–25). A key molecule in this process is the TCR \( \beta \) chain and the three ITAMs contained therein. Cross-linking of the \( \beta \) chain or constructs containing individual ITAMs alone were found to be sufficient to induce T cell activation and Fas-mediated apoptosis (28, 35, 36). In agreement with these findings, we have shown here that TCR-\( \beta \) as well as the functional integrity of the ITAM signaling motifs of \( \beta \) were required for HIV-mediated upregulation of FasL. However, these findings further implied that HIV targets the TCR \( \beta \) chain directly through a viral protein. To date, several lines of evidence indicated that the Nef

Figure 4. FasL upregulation by HIV requires the TCR \( \beta \) chain. FasL expression was assessed 48 h after infection with HIV (IIIB strain) by immunoprecipitation using an anti-FasL mAb (A) or by flow cytometry (B). Wild-type (WT) and TCR-\( \beta \) Jurkat are compared with the TCR-\( \beta \) cells stably transfected with CD16\( \beta \) or CD16\( \beta \mu \) (signaling-defective CD16\( \beta \)). The level of viral replication in Jurkat cells was comparable as determined by p24 assay (wild-type, 3.5 ± 0.5; TCR-\( \beta \), 3.8 ± 1.0; CD16\( \beta \), 3.8 ± 1.0; CD16\( \beta \mu \), 3.5 ± 0.25 ng/ml).

Figure 5. Induction of FasL expression by Nef or a Nef mutant (Nef.PXmu). Jurkat cells were transfected with CD8-Nef or CD8-Nef.PXmu (no binding to TCR-\( \beta \)) construct by electroporation and kept under neomycin selection for 2 wk. Outgrowing cells were selected for CD8 surface expression by Dynabeads and analyzed for FasL expression by FACS® as described above.

Figure 6. Upregulation of FasL by Nef requires TCR-\( \beta \). Transient cotransfection of a FasL promotor/luciferase reporter with an empty vector (cont.; negative control), Tax (positive control), Nef, and Nef.PXmu into Jurkat (WT), TCR-\( \beta \) Jurkat (TCR-\( \beta \)), and TCR-\( \beta \) cells reconstituted with CD16-\( \beta \) (TCR-\( \beta \)/Zeta). The expression of the reporter gene was determined as described in Materials and Methods. Bars, the mean ± SD of three experiments.
protein exerted such a role. First, Nef-mediated activation of T cells has been demonstrated in a number of reports (8–10). Second, expression of Nef in the cytoplasm of T cells interferes with early T cell signaling events emanating from the TCR–CD3 complex, including hypophosphorylation of TCR-ζ, whereas expression of a plasma membrane–associated form of Nef causes AICD in Jurkat cells (8). Third, a very aggressive form of Nef from SIV, SIV-YE-Nef, basically functions like an ITAM domain of TCR-ζ (38). Finally, SIV-induced upregulation of FasL in T cells depends on the expression of an intact Nef protein (22), and Nef from a lethal SIV strain (smmPBj14) alone can directly cause FasL upregulation (39). Thus, it appeared very likely that Nef acted at the level of the TCR. Indeed, our study confirms this assumption by showing that Nef can directly interact with the TCR-ζ chain.

Strong evidence for the interaction of Nef with ζ came from a second, surprising finding. In Jurkat cells lacking the TCR, binding of the Nef-associated serine kinase p62/NAK was abolished. Conversely, reconstitution of these cells with the ζ ITAM 2 and 3 restored binding of p62/NAK with Nef. Furthermore, the integrity of the ITAM motif appeared to be important, since mutation of the ζ ITAMs greatly reduced the effect. As shown previously, the p62/NAK kinase has to be activated in order to bind to Nef (32). These results suggest a dynamic interaction of Nef with the ζ ITAMs, ultimately resulting in the activation of p62/NAK, which in turn binds to Nef. In view of our and other studies, it is likely that activation of p62/NAK is part of Nef-mediated stimulation of T cell signaling pathways; however, at this point it is not clear whether p62/NAK has a role in the Nef-mediated upregulation of FasL. Notably, Nef binds to p62/NAK in cells lacking a TCR (31; e.g., COS cells). In these cells, the TCR-ζ chain may be functionally replaced by other receptors, possibly containing ITAMs. This would explain why Nef has effects in cells usually not infected by HIV (40; e.g., NIH 3T3 cells).

More recently, Howe et al. showed that Nef from SIV or HIV-2 associated with the TCR-ζ chain but failed to show an interaction with HIV-1 Nef (41). Our study differs from that of Howe et al. in at least two respects. First, we made constructs to target Nef to the plasma membrane where the TCR is located. Second, we have established functional consequence of the Nef–ζ interaction which may have relevance to the pathogenesis of HIV interaction.

Induction of cell death by HIV could be mediated by different viral proteins. Cross-linking of CD4 by HIVgp120 in the presence of Tat protein can induce FasL expression and apoptosis of uninfected T cells (42). Additionally, interaction of HIVgp120 with chemokine receptor CXCR4 on macrophages leads to death of CD8+ T cells mediated by TNF–TNFRII interaction (15). In this study, we report an additional important mechanism of HIV-mediated apoptosis, which may act to protect HIV-infected T cells from CTL attack by killing Fas+ viral-specific CTLs in the process (1). Nef can also downregulate MHC class I expression and protect the infected cells against killing by CTLs (2), or CD4 expression leading to loss of CD4 T cell function (2).

**Figure 7.** Model describing mechanisms of immune evasion mediated by the HIV nef gene. Nef is expressed in the early viral life cycle and, after myristoylation, associates with the plasma membrane where several protein interactions take place. Nef interacts with ζ, which leads to the activation of p62/NAK, which in turn causes the binding of p62/NAK to Nef. These events ultimately stimulate FasL expression, which may protect infected cells from CTL attack by killing Fas+ viral-specific CTLs in the process (1). Nef can also downregulate MHC class I expression and protect infected cells against killing by CTLs (2), or CD4 expression leading to loss of CD4 T cell function (2).
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