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Activation of the T-Cell Receptor Signaling Pathway by Nef from an Aggressive Strain of Simian Immunodeficiency Virus

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The Nef from a highly virulent strain of simian immunodeficiency virus (SIV), SIVpbj14, and a Nef from the traditional strain SIVmac239 bearing the mutation from RQ to YE (YE-Nef) both induce an acute lethal disease in monkeys. The YE mutation and its surrounding sequence resemble the immunoreceptor tyrosine-based activation motif (ITAM), which is present in the cytoplasmic tail of T- and B-cell antigen receptors and mediates signaling during lymphocyte activation. We show here that the ITAM from YE-Nef performs the same function. First, not only does YE-Nef increase the activity of the transcription factor NFAT, which is one of the downstream targets of T-cell activation, but the ITAM from the YE-Nef by itself also activates NFAT. Second, the ITAM from YE-Nef is phosphorylated on tyrosine residues by Lck and associates with ZAP-70, a T-cell-specific tyrosine kinase. The phosphorylation of both conserved tyrosine residues on the ITAM is required for the recruitment of ZAP-70. Finally, Lck is required for the activation of NFAT by YE-Nef. These results demonstrate that YE-Nef contains a functional ITAM and elucidate the molecular mechanisms underlying the pathogenesis of SIVpbj14.

Nef is a 25- to 35-kDa viral protein unique to the primate lentiviruses human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV) and is encoded by the open reading frame overlapping the 3′ long terminal repeat (19, 37). Nef is incorporated into virions (34, 43, 50) and accounts for 85% of the earliest-expressed viral proteins in infected cells (38). Nef is myristoylated at its conserved N-terminal glycine, and the myristoylation of Nef is critical for its function and is required to direct Nef to cellular membranes (19, 37). Three-dimensional studies of HIV Nef demonstrated that Nef consists of two domains: a disordered N-terminal anchor domain and a compactly folded C-terminal core domain (18, 27).

Many studies demonstrated that Nef is critical for maintaining high levels of viral replication and for the pathogenesis of AIDS. SIV expressing a truncated Nef does not lead to viremia and death in rhesus macaques, and reversion of this Nef mutation restores high levels of viremia and progression of disease (25). In addition, mutations and deletions in the *nef* gene have been detected in HIV-1 strains isolated from long-term survivors (9). Nef of HIV-1 is also important for viral replication and pathogenesis in the SCID/hu mouse model (24). Moreover, the important role of Nef in AIDS pathogenesis is supported by the observation that development of $CD4^+$ cells is perturbed in transgenic mice which express Nef in T cells (3, 29, 44).

Although the biological importance of Nef has been proven, the function of Nef at the molecular level remains poorly understood. Nef (negative factor) was initially reported to be a negative regulator of viral gene expression and was dispensable for viral replication in tissue culture. However, subsequent studies demonstrated that Nef can enhance viral replication and viral infectivity (19, 37). Nef also decreases the expression of CD4, the primary receptor for HIV and SIV, on the cell surface. Down-regulation of CD4 may prevent super infection of infected cells and impair normal T-cell functions (19, 37).

Much evidence indicates that Nef interacts with cellular proteins involved in signal transduction. Nef from various HIV or SIV strains contains a highly conserved PXXP sequence, which is the consensus binding site for the SH3 domain of Src family kinases (39). Studies from different laboratories demonstrated that Nef can also interact with cellular kinases other than Src family kinases, including phosphatidylinositol 3-kinase, protein kinase C, mitogen-activation protein kinase, and a 62-kDa PAK-related serine/threonine kinase (8, 16, 41, 42, 45). Nef has also been shown to alter the activation stage of T cells, but it remains controversial whether Nef activates or inhibits T-cell signaling (19, 37). Using a CD8-Nef fusion protein, we demonstrated previously that membrane expression of Nef induces T-cell activation (1).

Recently, Du and coworkers found a *nef* allele from SIVpbj14 that also induces lymphocyte activation (11). SIVpbj14, an unusual strain of SIV, can replicate well in resting peripheral blood mononuclear cells (PBMCs), while other viruses can replicate only in activated PBMCs (13). SIVpbj14 infects pig-tailed macaques and induces an unusually rapid and severe disease which results in death within 10 days (14). Interestingly, a similar phenotype can be created by RQ-to-YE mutations on residues 17 and 18 of Nef (YE-Nef) from SIVmac239 (11). The YE mutation and its surrounding sequence on SIV Nef resemble a conserved domain called the immunoreceptor tyrosine-based activation motif (ITAM). The ITAM, which has the consensus sequence $YXXLX_{6-8}YXXL$, is present in the intracellular domain of the T-cell receptor (TCR) and B-cell receptor (BCR) is an essential signaling motif for lymphocyte activation (5, 21). Du et al. also showed that both tyrosine residues on the putative ITAM from YE-Nef are required for viral replication in unstimulated PBMCs (10). Interestingly, an ITAM is also present in the cytoplasmic tail of two viral proteins, the latent membrane protein LMP2A of Epstein-Barr virus and the envelope protein gp30 of bovine leukemia virus, and these motifs alter the activation stage of lymphocytes to

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facilitate viral replication (2, 30, 51). However, there is no evidence that the putative ITAM from YE-Nef is indeed a functional ITAM, and its mechanism of action remains to be elucidated.

In this study, we used transcriptional assays to demonstrate that the putative ITAM from YE-Nef is sufficient to activate T-cell signaling. Various biochemical and genetic approaches also revealed the molecular mechanism of this ITAM from YE-Nef in T-cell activation.

MATERIALS AND METHODS

Constructs. Plasmid NFAT-Luciferase, a luciferase reporter driven by a synthetic promoter containing multiple NFAT binding sites, was a generous gift from Gerald Crabtree, Stanford University. Plasmid pEF-Nef was obtained by subcloning the *nef* gene of SIVmac239 into the expression vector pEF-BOS (31). Nef was also fused with the extracellular and transmembrane portion of CD8, and the CD8-Nef hybrid was also subcloned into pEF-BOS, resulting in pEF-CD8-Nef-myc (CN). The mutations on amino acids 17 and 18 (RQ to YE) were introduced into the *nef* gene on these constructs by PCR-mediated mutagenesis, resulting in pEF-BOS-YE-Nef (YE-Nef) and pEF-BOS-CD8-YE-Nef (CYEN). The CD8 hybrids with the truncated Nef or YE-Nef that contains only the N-terminal 43 amino acids were obtained also by PCR, and they were also subcloned into pEF-BOS, generating pEF-CD8-Nef.43 (CN.43) and pEF-CD8-YE-Nef (CYEN.43). CD8 hybrids with the CD3 ζ chain which contains three ITAM motifs were also expressed from pEF-BOS. The nucleotide sequence of each Nef construct was confirmed by the dideoxy-chain termination method. Plasmids used in transfections were prepared by two rounds of equilibrium centrifugation in CsCl-ethidium bromide gradients. Human ZAP-70, Lck, and kinase-inactive Lck (Lck-A273) were expressed from the expression vector pSM (23) (generous gifts from Arthur Weiss, University of California San Francisco). The plasmids expressing murine Lck, Src, and Fyn were kindly provided by Cliff Lowell, University of California San Francisco.

Cell lines and transfection. Human leukemia T-cell line Jurkat and its derivative JCAM1 (46), a Lck-kinase deficient cell line, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.2 mM glutamate, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. COS-7, a monkey kidney cell line, was grown in similarly supplemented Dulbecco's modified Eagle's medium. JCAM/Lck cells were derived from JCAM1 cells that stably express Lck kinase (46) (from Arthur Weiss), and they are maintained in complete medium containing 100 μg of hygromycin per ml.

Jurkat and derivative cells were transfected by electroporation. For each transfection, 10^7 cells were resuspended in 0.4 ml of serum-free medium and mixed with 20 μg of plasmid NFAT-Luciferase and 20 μg of effector plasmids at room temperature. The mixture was electroporated at 250 V and 960 μF with a Gene Pulser (Bio-Rad, Hercules, Calif.), and the transfected cells were grown in 10 ml of complete medium at 37°C under 5% CO_2. Every 100-mm-diameter dish of COS-7 cells was transfected with a total of 10 μg of the indicated plasmids and 30 μl of Lipofectamine (GIBCO, Grand Island, N.Y.) as the manufacturer suggests.

Luciferase assays. Transfected Jurkat and derivative cells were aliquoted into a 96-well U-bottom plate (2×10^5 cells/100 μl of medium/well) 24 h after transfection. Cells were unstimulated or stimulated with 50 ng of phorbol myristate acetate and 1 μM ionomycin. After 6 to 8 h of incubation, cells were lysed in 10 μl of lysis buffer (100 mM KPO_4 [pH 7.8], 1 mM dithiothreitol, 1% Triton X-100) for 5 min and mixed with 100 μl assay buffer (200 mM KPO_4 [pH 7.8], 10 mM ATP, 20 mM $MgCl_2$). Luciferase activities were determined by using a MicroLumat LB96P (Berthold, Bad Wildbad, Germany), and each experimental condition was assayed in triplicate.

Immunoprecipitation. Transfected COS-7 cells were washed with phosphate-buffered saline (PBS) twice and lysed in 1 ml of lysis buffer containing 1% Nonidet P-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors at 4°C for 20 min. The cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatants were incubated with 1 to 2 μl of anti-CD8 antibodies at 4°C for 1 h. The samples were then mixed with 40 μl of protein A-conjugated agarose beads for another hour, and the immunoprecipitates were washed four times and resuspended in sodium dodecyl sulfate (SDS) sample buffer.

Immunoblotting. Protein samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel and transferred to nitrocellulose, using a Bio-Rad Western blotting apparatus. Membranes were blocked by PBS containing 5% nonfat milk for an hour and incubated with primary antibodies for at least an hour. After three washes with PBS containing 0.1% Tween 20, membranes were incubated with a secondary antibody conjugated with horseradish peroxidase for 40 min and then analyzed by an enhanced chemiluminescence assay (ECL kit; Amersham, Arlington Heights, Ill.). The stripping and reprobing were carried out according to the instructions for use of the ECL kit.

Antibodies. OKT8 (monoclonal antibody [MAb] against human CD8) and 2F3.2 (MAb against human ZAP-70) were generous gifts from Arthur Weiss. The MAb against Lck was provided by Joseph Bolen (DNAX, Palo Alto, Calif.). 4G10 (MAb against phosphotyrosine) was purchased from Upstate Biotechnology, Inc., Lake Placid, N.Y. The polyclonal antibody against SIV Nef was a generous gift from Earl Sawai, University of California, Davis. The anti-Myc MAb was purchased from Calbiochem, La Jolla, Calif.

Flow cytometry. A total of 5×10^5 to 10^6 transfected cells were stained by fluorescein isothiocyanate (FITC)-conjugated anti-Leu2a (anti-CD8) (Becton Dickinson, Mountain View, Calif.) and then analyzed with a FACScan (Becton Dickinson).

Peptide and peptide binding assays. Biotin-conjugated peptides with the sequence GDLYERLLRARGETYGRLLGE, which represents the sequence from residues 14 to 33 of YE-Nef, were synthesized by a peptide synthesizer (Applied Biosystems Inc., Foster City, Calif.). These peptides were either unphosphorylated (Nef-ITAM-OP) or phosphorylated at tyrosine corresponding to residue 17 of YE-Nef (Nef-ITAM-NP), Y at residue 28 (Nef-ITAM-CP), or both tyrosine residues (Nef-ITAM-2P). Indicated amounts of peptides were mixed with 500 μl of whole-cell lysates from 10^7 resting Jurkat cells. After incubation at 4°C for 2 h, the samples were incubated with avidin-agarose beads (Vector Laboratories, Burlingame, Calif.) for 1 h. Peptide precipitate was washed with lysis buffer four times and resuspended in SDS sample buffer.

RESULTS

The ITAM from YE-Nef activates Jurkat cells. Since the sequence of the ITAM from YE-Nef is virtually identical to its corresponding sequence in SIVpbj14 except for the substitution of amino acid Q by R at position 22 (11), YE-Nef was used in this study. Moreover, this YE-Nef caused acute disease in monkeys (11). Finally, YE-Nef and Nef also differ by only two amino acids in the ITAM, and so the effects of YE-Nef could be compared directly to those of the wild-type Nef from SIVmac239. To determine if the YERLLRARGETYGRL sequence from YE-Nef represents a functional ITAM, we used the NFAT assay to study its effects on T-cell activation. NFAT (nuclear factor of activated T cells) is a T-cell-specific transcription factor whose activity is greatly enhanced during T-cell activation. Thus, the luciferase gene under the control of a synthetic promoter containing four NFAT binding sites (plasmid NFAT-Luciferase) has been used as a convenient reporter for T-cell activation (52). The *nef* gene from SIVmac239 and the *YE-nef* gene bearing mutations in amino acids 17 and 18 (RQ to YE) were subcloned into the expression vector pEF-BOS (Fig. 1A), and these plasmids were cotransfected with NFAT-Luciferase into Jurkat cells, which are human leukemic T cells. Similar levels of Nef and YE-Nef proteins were expressed in cotransfected cells (data not shown). In luciferase assays, YE-Nef caused a fourfold increase in NFAT activity compared to Nef (Fig. 1B).

CD8 fusion proteins have been used extensively to characterize the function of ITAMs from TCR, BCR, LMP2A of Epstein-Barr virus, and gp30 of bovine leukemia virus (1, 2, 22, 41). Therefore, we also analyzed the putative ITAM from YE-Nef by fusing Nef and YE-Nef proteins to the extracellular and transmembrane domains of CD8 (Fig. 1). Plasmid CD8-Nef (CN) or CD8-YE-Nef (CYEN) was cotransfected with NFAT-Luciferase into Jurkat cells, and Western blotting revealed that similar levels of CN or CYEN protein were expressed in transfected cells (data not shown). The surface expression of chimeric proteins were also quantitated by fluorescence-activated cell sorting (FACS) analyses with anti-CD8 FITC staining, which demonstrated that slightly more CYEN than CN was expressed on the cell surface (Fig. 1C). The low surface expression of CD8 fusion proteins is caused by the rapid internalization of Nef (1, 53). Luciferase assays demonstrated 18-fold-higher NFAT activities in cells expressing CYEN than in those expressing CN (Fig. 1B). To examine if the ITAM of YE-Nef was sufficient to induce this increased NFAT activity, CD8 was fused to the truncated Nef that contained only the N-terminal 43 amino acids of Nef or YE-Nef with the intact ITAM (CN.43 and CYEN.43) (Fig. 1). FACS

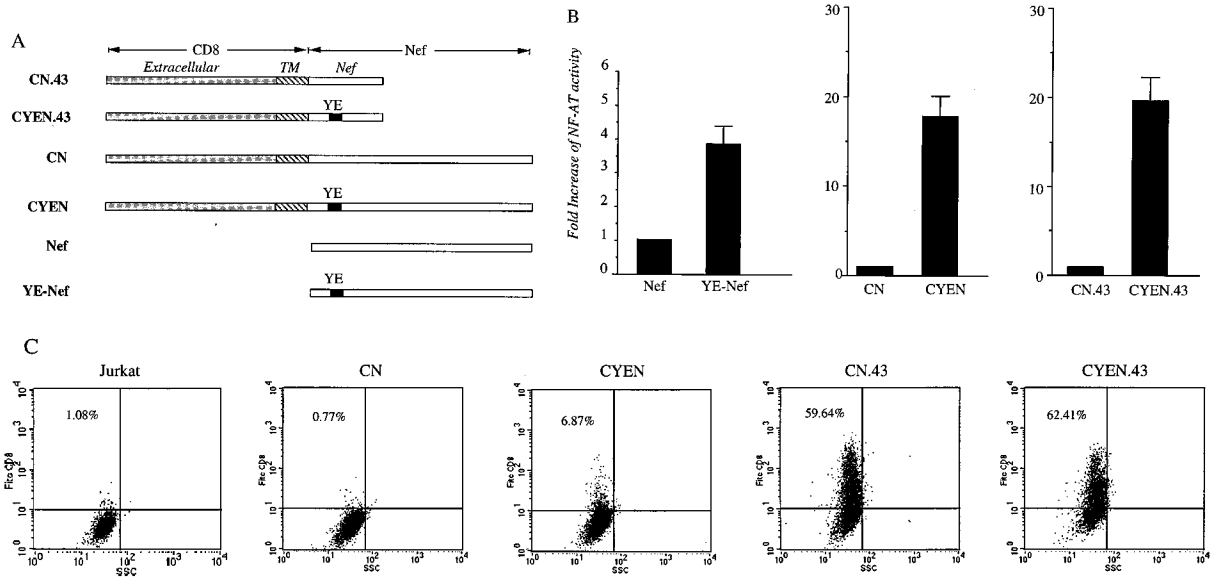

FIG. 1. YE-Nef activates NFAT in Jurkat cells. (A) Schematic representation of Nef plasmid construction. The wild-type SIVmac239 Nef sequence is shown by open bars, and the YE mutation is represented by black boxes. Shorter fragments in CN.43 and CYEN.43 represent the N-terminal 43 residues of Nef, and longer fragments correspond to full-length Nef proteins. The extracellular and transmembrane portions of human CD8 are depicted as gray and hatched boxes, respectively. These fragments were subcloned into the mammalian expression vector pEF-BOS. Names of plasmids are given on the left. (B) Various Nef plasmids were cotransfected with plasmid NFAT-Luciferase into Jurkat cells, and luciferase assays were performed 1 day later. Results are shown as increased NFAT activity of YE-Nef over Nef, whose activity was arbitrarily set to 1. These results are averages of at least three independent experiments, and the error bars represent standard errors of the mean. (C) Expression of the CD8 epitope at the surface of the untransfected Jurkat cells and Jurkat cells transfected with CD8 fusion proteins. Transfected Jurkat cells were stained with the FITC-conjugated antibody anti-Leu2a and analyzed by FACS. Plasmids used in transfections are shown at the top, and the percentage of CD8-positive cells is shown in the upper left quadrant. SSC, side scatter.

analyses exhibited similar surface expression patterns of CN.43 and CYEN.43 in transfected cells (Fig. 1C). The results from NFAT assays showed 20-fold-higher NFAT activities in Jurkat cells expressing CYEN.43 than in Jurkat cells expressing CN.43 (Fig. 1B). This activation was comparable to the NFAT activity induced by the hybrid CD8 protein fused to the cytoplasmic portion of the TCR ζ chain which contains three ITAMs (Cζ) (data not shown). These results strongly suggest that the putative ITAM from YE-Nef is a functional ITAM.

Tyrosine-phosphorylated ITAM from YE-Nef binds to ZAP-70 in lysates from Jurkat cells. In T cells, stimulation of TCR triggers the phosphorylation of both tyrosine residues on the ITAM from the TCR, and the phosphorylated ITAM provides the binding site for a T-cell-specific tyrosine kinase ZAP-70, which subsequently activates downstream signaling cascades (5, 21). To test whether a similar sequence of events occurs with the ITAM from YE-Nef, we used synthetic biotinylated peptides representing the ITAM from YE-Nef in the ZAP-70 binding assay. These peptides were either unphosphorylated or phosphorylated on residue 17Y, residue 28Y, or both. Five micrograms of each peptide was incubated with Jurkat cell lysates and precipitated by avidin-conjugated agarose beads. Western blotting using an antibody against ZAP-70 showed that a 70-kDa protein corresponding to ZAP-70 bound to the doubly phosphorylated peptides representing the ITAM from YE-Nef but did not interact with singly phosphorylated or unphosphorylated peptides (Fig. 2). The intensity of the ZAP-70 band was decreased when lower concentrations of the doubly phosphorylated peptide were used in the binding assay (Fig. 2). This result demonstrates that ZAP-70 specifically binds to the doubly phosphorylated ITAM from YE-Nef in a dose-dependent manner.

Src family kinases phosphorylate YE-Nef, which leads to its association with ZAP-70 in Cos cells. Since ZAP-70 binds to the phosphorylated ITAM from YE-Nef in Jurkat cells, we wanted to determine which tyrosine kinase is responsible for this phosphorylation. A previous study demonstrated that Src was able to associate with and phosphorylate YE-Nef when it was expressed with YE-Nef in NIH 3T3 cells (11). However, due to its low levels of expression, Src did not seem to be the physiological partner for YE-Nef in T cells. Two other Src family kinases, Fyn and Lck, which are expressed at high levels in T lymphocytes were more likely candidates. To examine if Fyn and Lck are able to phosphorylate the ITAM from YE-Nef, plasmids expressing Src, Fyn or Lck, and ZAP-70 were cotransfected with CN or CYEN into COS cells which have no endogenous expression of Lck and Fyn. Since Cζ protein can be phosphorylated by Fyn and Lck and associate with ZAP-70

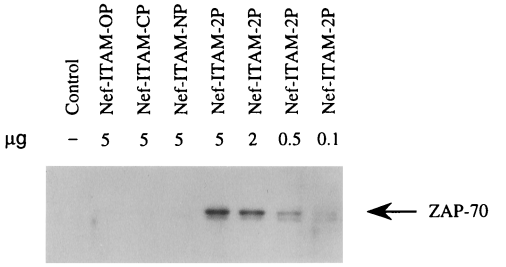

FIG. 2. The ITAM peptide from YE-Nef binds to ZAP-70 in Jurkat cells. Various biotin-conjugated peptides representing the sequence from residues 14 to 33 of the YE-Nef which contains the intact ITAM are shown at the top. Indicated amounts of biotin-conjugated peptides representing the ITAM sequence from YE-Nef were mixed with whole-cell lysates from Jurkat cells, and these peptides were isolated by avidin-agarose beads. Avidin-conjugated beads with no peptide were used as the control. Samples were analyzed by SDS-PAGE (10% gel) and immunoblotted with the antibody against ZAP-70.

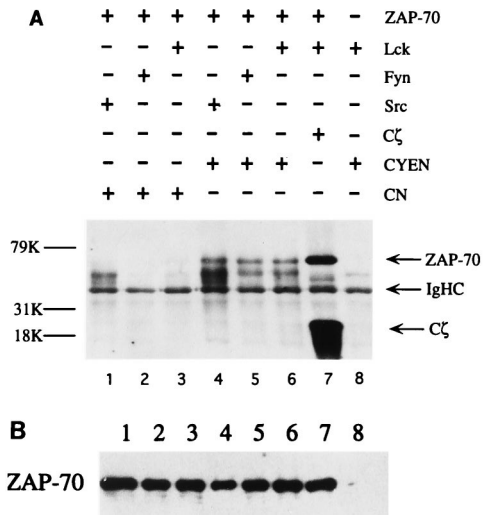

FIG. 3. ZAP-70 and Src family kinases associate with YE-Nef in COS cells. CN (lanes 1 to 3) or CYEN (lanes 4 to 6 and 8) was cotransfected with plasmids expressing ZAP-70 (lanes 1 to 7) and Src (lanes 1 and 4), Fyn (lanes 2 and 5), or Lck (lanes 3 and 6 to 8) into COS cells. Plasmids used in each transfection are indicated by plus signs at the top. In lane 8, the empty vector lacking ZAP-70 was used in the cotransfection. The plasmid encoding Cζ was also cotransfected with ZAP-70- and Lck-expressing plasmids (lane 7). (A) Transfected cells were lysed and immunoprecipitated with an anti-CD8 antibody. Samples were analyzed by SDS-PAGE (10% gel) and subjected to Western blotting with the antiphosphotyrosine MAb 4G10. Sizes of the molecular marker are shown on the left. The immunoglobulin heavy chain (IgHc), tyrosine-phosphorylated ZAP-70, and Cζ proteins are indicated by the arrows on the right. (B) Expression levels of ZAP-70 in each transfection were analyzed by immunoblotting of whole-cell lysates from transfected cells with an anti-ZAP-70 antibody.

(7, 23), a Cζ-expressing plasmid was used in the cotransfection experiment as the positive control (Fig. 3A, lane 7). Coimmunoprecipitation assays demonstrated that CYEN and Cζ, but not CN, associate with a 70-kDa phosphorylated protein (Fig. 3A). This 70-kDa protein was absent in cells cotransfected with the plasmid vector lacking ZAP-70, suggesting that it was ZAP-70 (Fig. 3A, lane 8). The expression levels of ZAP-70 in these transfected cells were also analyzed by Western blotting using antibodies against ZAP-70 (Fig. 3B). These results suggest that ZAP-70 specifically binds to YE-Nef in the presence of Src family kinases. A 60-kDa tyrosine-phosphorylated protein also coimmunoprecipitated with CN and CYEN in cells expressing Src (Fig. 3A, lanes 1 and 4), suggesting that it represents Src. Similarly, the 60-kDa phosphoproteins which associate with CYEN in cells expressing Lck or Fyn represent these tyrosine kinases (Fig. 3A, lanes 5 and 6). Since CN and CYEN comigrate with the heavy chain of immunoglobulin, the tyrosine phosphorylation of CN and CYEN could not be revealed in this blot (Fig. 3A).

To examine if the ITAM from YE-Nef can mediate the interaction with ZAP-70, CN.43 and CYEN.43 were also tested in similar coimmunoprecipitation assays. Immunoblots with the antibody against ZAP-70 revealed that a 70-kDa protein coimmunoprecipitated with CYEN.43, but not CN.43, in the presence of Lck (Fig. 4A, lanes 1 and 2). Immunoblotting of the same membrane with the antiphosphotyrosine antibody indicated that CYEN.43 also became tyrosine phosphorylated (Fig. 4B, lane 2). However, CYEN.43 was not phosphorylated and failed to interact with ZAP-70 in cells expressing the kinase-inactive form of Lck that contains a mutation of K to A at position 273 (Fig. 4A and B, lanes 3). Thus, the kinase activity of Lck is required for the phosphorylation of the ITAM from YE-Nef and its interaction with ZAP-70. Note that the phosphorylation of CYEN.43 in cells expressing the parental plasmid vector alone was decreased compared to that in cells coexpressing ZAP-70 (Fig. 4B, lane 4), which was caused by the lack of protection of phosphotyrosine from the cellular tyrosine phosphatase by ZAP-70 SH2 domains, as observed previously (23). The same membrane was also subjected to Western blotting with the antibody against SIV Nef, demonstrating that comparable amounts of CD8 fusion proteins were present in each immunoprecipitated complex (Fig. 4C). Analysis of total cell lysates from these transfected cells confirmed that comparable levels of Lck and ZAP-70 were expressed (Fig. 4D).

Lck is required for T-cell signaling mediated by YE-Nef. We have shown that the ITAM from YE-Nef is sufficient to activate T-cell signaling in Jurkat cells and that the tyrosine phosphorylation of this ITAM is critical for this event. Although the ITAM from YE-Nef can be phosphorylated in COS cells by several Src family kinases, the role of these kinases in T-cell activation mediated by YE-Nef had to be tested directly in T lymphocytes. For instance, both of Fyn and Lck have been shown to phosphorylate the ITAM of the ζ chain of TCR, but TCRζ is preferentially phosphorylated by Lck, but not Fyn, in

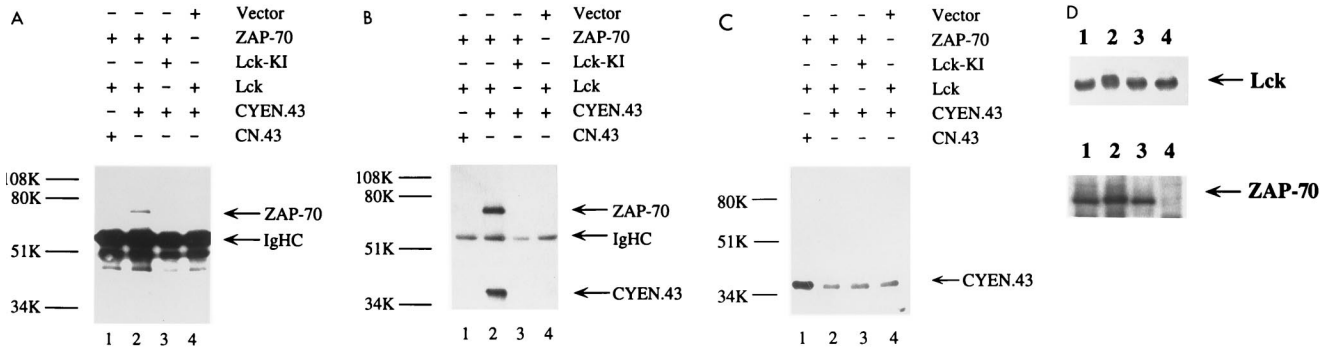

FIG. 4. Association of ZAP-70 with the ITAM from YE-Nef. CN.43 (lane 1) or CYEN.43 (lanes 2 to 4) was cotransfected with plasmids expressing Lck (lanes 1, 2, and 4) and ZAP-70 (lanes 1 to 3) into COS cells. CYEN.43 was also transfected with the plasmid expressing kinase-inactive Lck (lane 3) or the empty vector (lane 4). Plasmids used in each transfection are indicated by plus signs at the top. (A) Transfected cells were lysed, CD8 fusion proteins were immunoprecipitated by the antibody against CD8, and samples were then subjected to Western blotting with an anti-ZAP-70 antibody. The same blot was stripped and reprobed with the antiphosphotyrosine antibody 4G10 (B) or the antibody against SIV Nef (C). The immunoglobulin heavy chain (IgHc), ZAP-70, and tyrosine-phosphorylated CYEN.43 are indicated by the arrows on the right. (D) Amounts of ZAP-70 and Lck from each transfection were analyzed by immunoblotting whole-cell lysates with anti-ZAP-70 and anti-Lck antibodies as indicated.

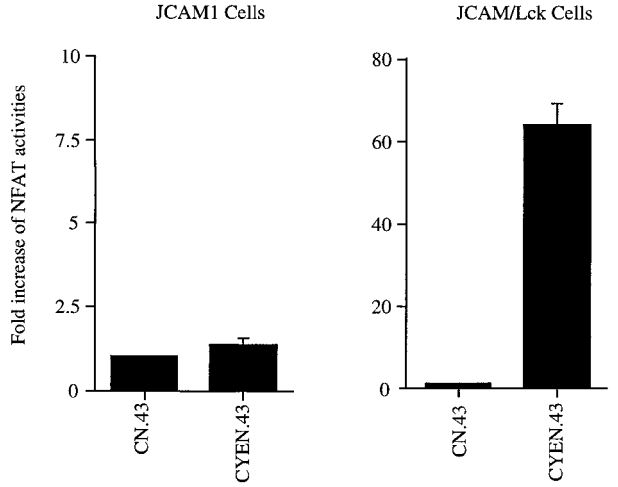

FIG. 5. Lck is required for the activation of NFAT by YE-Nef. Various Nef plasmids were cotransfected with plasmid NFAT-Luciferase into JCAM1 or JCAM1-Lck cells. After 24 h of incubation, cells were lysed and assayed for luciferase activity. Results are shown as fold increased NFAT activity of CYEN.43 over CN.43 or CYEN over CN plasmids. The results are averages of three independent experiments, and the error bars represent standard errors of the mean.

T cells (46, 48). Thus, we decided to use JCAM1 cells, which are mutant Jurkat cells deficient in Lck (46), to examine the ability of the ITAM from YE-Nef to activate NFAT in the absence of Lck. The NFAT activity in JCAM1 cells expressing CYEN.43 was the same as in Jurkat cells expressing CN.43 (Fig. 5), suggesting that the ITAM from YE-Nef failed to activate T-cell signaling without the functional Lck. Consistent with this result, increased NFAT activity was induced by CYEN.43 compared to CN.43 in JCAM1/Lck cells (46), which are JCAM1 cells that were reconstituted with the functional Lck (Fig. 5). Since FACS analyses showed similar levels of expression of CN.43 and CYEN.43 in JCAM1 or JCAM/Lck cells (data not shown), these effects were not due to the altered expression of CD8 fusion proteins in these cells. These data indicate that Lck, but not Fyn, is involved in T-cell activation by the ITAM from YE-Nef.

DISCUSSION

Various biochemical and genetic approaches were used to characterize the role of the YERLLRARGETYGRL sequence from YE-Nef in T-cell signaling. First, YE-Nef activated the transcriptional activity of NFAT, and the putative ITAM of YE-Nef was sufficient to mediate this event. This putative ITAM sequence, when doubly phosphorylated on tyrosine residues, could associate with ZAP-70. The tyrosine phosphorylation of the putative ITAM of YE-Nef could be achieved by several Src family kinases tested, including Src, Fyn, and Lck. Furthermore, JCAM1 cells demonstrated that Lck rather than Fyn was required for this T-cell activation. Therefore, the putative ITAM from YE-Nef, and thus from Nef of SIVpbj14, is indeed a functional ITAM, which explains the profound cellular activation by this Nef allele.

In the NFAT assay, CD8 Nef chimeras were able to activate T-cell signaling in the absence of cross-linking. This may have been caused by the dimeric nature of CD8 and Nef (12, 20, 26, 53) and the aggregation of the chimeras on the cell surface by the endogenous class I major histocompatibility complex determinants on Jurkat cells. In addition, levels of expression achieved in transient expression assays are higher than those observed in stable cell lines.

The interaction between ZAP-70 and YE-Nef was mediated by the ITAM. This observation was proved by the binding of doubly phosphorylated ITAM peptide to ZAP-70 and coimmunoprecipitation of ZAP-70 with the N-terminal 43 amino

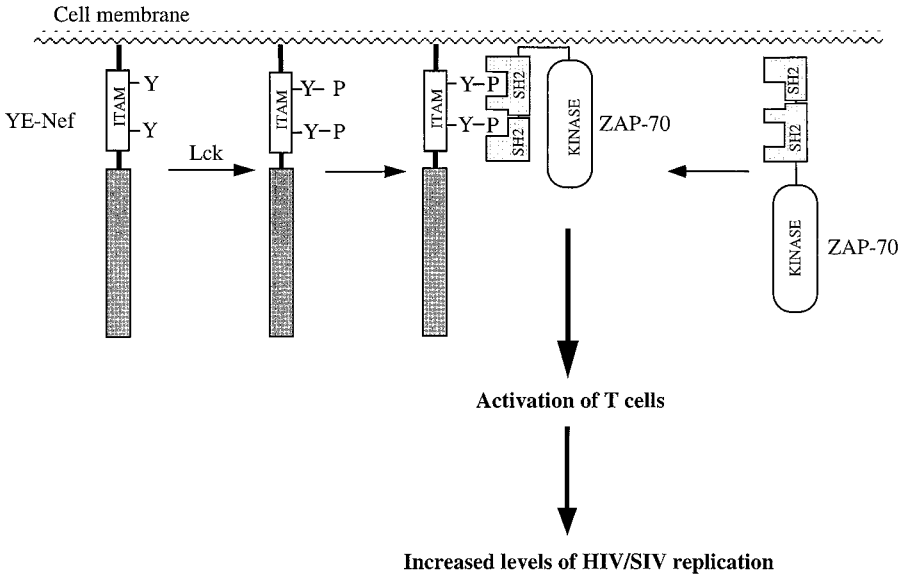

FIG. 6. The mechanism of T-cell activation by YE-Nef. The myristoylated YE-Nef is localized to cellular membranes and phosphorylated by Lck on both tyrosine residues of the ITAM, to which ZAP-70 binds via its two SH2 domains. This leads to the activation of ZAP-70, downstream T-cell signaling cascades, and increased levels of viral replication. Steps in this activation are diagrammed from left to right. Tyrosine residues (Y) are phosphorylated (P) in the ITAM. Two SH2 domains (gray rectangular structures) bind to the ITAM in the C-to-N-terminal direction (note the change in the conformation of ZAP-70 upon binding to the phosphorylated tyrosine residues in the ITAM). ZAP-70 itself is further phosphorylated and activates downstream effector proteins via its kinase domain. Consequences to the virus could be increased rates of initiation of transcription, optimized expression and assembly of viral proteins, and incorporation of cellular kinases into the virion, which would lead to posttranslational modification of viral proteins.

acids of Nef containing the intact ITAM. The peptide binding assay also revealed that both tyrosine residues on the ITAM of YE-Nef must be phosphorylated for its association with ZAP-70, suggesting that each of the phosphotyrosine residues interacts with one of the two SH2 domains of ZAP-70 (36). No intermediate proteins were detected in either case, indicating that ZAP-70 binds directly to the ITAM from YE-Nef. Thus, the ITAM from YE-Nef behaves identically to the ITAM from the ζ chain of TCR.

JCAM1 cells were used to study the significance of Lck and Fyn in YE-Nef-mediated signaling. The biochemistry of JCAM1 cells has been well characterized (46). These cells are deficient in Lck but still express Fyn. Although the overexpression of Fyn can phosphorylate YE-Nef in COS cells, Fyn could not substitute for the loss of Lck kinase activity to phosphorylate the ITAM in JCAM1 cells (32, 46). This phenomenon may be due to differences in level of expression, intracellular distribution, or substrate specificity of Fyn and Lck (17, 28, 33, 35). Similar observations have been made for the ability of Lck and Fyn to phosphorylate TCRζ on tyrosine residues (7, 46, 48).

The present work supports and extends the observation by Du et al. (11) and provides the molecular mechanism for the ability of YE-Nef and Nef from SIVpbj14 to activate infected cells. The previous work also indicated that macrophages are required for the replication of SIVpbj14 in resting PBMCs (11). However, the function of macrophages in this process remains unclear. Since T-cell activation involves two distinct signals, an antigen-specific signal via the TCR and a costimulatory signal provided by antigen-presenting cells, our results suggest that YE-Nef mimicks the TCR and the costimulatory signal might be provided by macrophages via costimulatory receptors.

Based on our study and studies by others on the ITAM from TCR (36, 49), we present a model on how YE-Nef activates T-cell signaling (Fig. 6). After its targeting to the cellular membrane, two tyrosine residues of the ITAM from YE-Nef are phosphorylated by Lck. The doubly tyrosine phosphorylated ITAM recruits ZAP-70 via its two SH2 domains, and this leads to the activation of ZAP-70 and the T-cell signaling cascade, which facilitates efferent and afferent pathways of viral replication. For example, NFAT has been shown to activate HIV-1 replication and gene expression in T cells (22). ERK2, which has been recently shown to be incorporated into virions, can also be activated via T-cell activation (6). In addition, the association of YE-Nef with ZAP-70 and Src family kinases may also transport and package tyrosine kinases into virions, where they might phosphorylate viral components. The phosphorylation of viral protein, e.g., matrix, is important for the release of reverse transcriptase complexes from membrane and for nuclear targeting of the viral DNA in nondividing cells (4, 15, 47). Moreover, extensive lymphocyte and macrophage activation might be sufficient to account for the toxic shock-like picture of the acute infection observed with SIVpbj14 (11, 40).

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