Human Immunodeficiency Virus Type 1 Nef Protein Inhibits Activation Pathways in Peripheral Blood Mononuclear Cells and T-Cell Lines

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Human immunodeficiency virus type 1 (HIV-1) Nef protein causes the loss of cell surface CD4 and interleukin-2 (IL-2) receptor (Tac) from peripheral blood mononuclear cells (PBMC) and CD4⁺ T-cell lines. As both CD4 and the IL-2 receptor play crucial roles in antigen-driven helper T-cell signalling and T-cell proliferation, respectively, the role of Nef in the viral life cycle may be to perturb signalling pathways emanating from these receptors. However, the intracellular targets for Nef that result in receptor down-regulation are unknown. Using a recombinant glutathione *S*-transferase–full-length 27 kDa Nef (Nef 27) fusion protein, produced in *Escherichia coli* by translation from the first start codon of HIV-1 *nef* clone pNL4-3, as an affinity reagent to probe cytoplasmic extracts of MT-2 cells and PBMC, we have shown interaction with at least seven host cell protein species ranging from 24 to 75 kDa. Immunoblotting identified four of these proteins as p56^{lck}, CD4, p53, and p44^{mapk/erk1}, all of which are intimately involved in intracellular signalling. To assess the relevance of these interactions and further define the biochemical activity of Nef in signal transduction pathways, highly purified Nef 27 protein was introduced directly into PBMC by electroporation. Nef 27-treated PBMC showed reduced proliferative responsiveness to exogenous recombinant IL-2. Normally, stimulation of T-cells by IL-2 or phorbol 12-myristate 13-acetate provokes both augmentation of p56^{lck} activity and corresponding posttranslational modification of p56^{lck}. These changes were also inhibited by treatment of PBMC with Nef, suggesting that Nef interferes with activation of p56^{lck} and as a consequence of signalling via the IL-2 receptor. Further evidence for Nef interfering with cell proliferation was the decreased production of the proto-oncogene *c-myc*, which is required for cell cycle progression, in Nef-treated MT-2 cells. In contrast to the binding characteristics and biological effects of Nef 27, the alternate 25-kDa isoform of Nef (Nef 25) produced by translation from the second start codon of HIV *nef* pNL4-3 (57 nucleotide residues downstream) was shown to interact with only three cellular proteins of approximately 26, 28, and 56 kDa from PBMC and MT-2 cells, one of which was identified as p56^{lck}. Also, proliferation and posttranslational modification of p56^{lck} in response to IL-2 stimulation were not profoundly affected by treatment of PBMC with Nef 25 compared with Nef 27. However, Nef 25 did exhibit some activity since treatment of MT-2 cells with Nef 25 resulted in decreased expression of *c-myc*. The effect of HIV-1 Nef on cell activation and proliferation may partly be explained by its interaction with specific cellular proteins. Interaction of Nef with these proteins may modulate their activity such that the cellular response to antigen or cytokines is severely altered resulting in the profound immunodeficiency characteristic of HIV infection.

Human immunodeficiency virus type 1 (HIV-1) contains several accessory genes in addition to the structural genes, *gag*, *pol*, and *env* (for a review, see reference 12). The products of some of these genes have clearly defined functions, whereas others, such as Nef, are less well understood (11, 12, 54). It is known that the *nef* gene product, expressed early during infection from one of the major 1.8-kb mRNA species (41), is not required for HIV-1 replication and cytopathic effects in vitro (28). Earlier studies performed with *nef*-deleted viruses indicated more efficient replication than their parent wild type (2, 9, 28, 39, 54). More recent studies in primary cell types indicate the reverse to be true (31, 51). Certainly, in vivo data with molecular clones of the related simian immunodeficiency virus show *nef* to be essential for maintenance of high virus load and the development of acquired immunodeficiency (24). Since the *nef* gene is highly conserved among strains of HIV-1, HIV-2,

and simian immunodeficiency virus (47), it is likely that the expressed protein plays an important role in the virus life cycle.

Nef is a small 25- to 30-kDa polypeptide which is modified by N-terminal myristoylation and, presumably as a consequence of this myristoyl anchor, is associated predominantly with the plasma membrane (14, 19, 23). Apart from its effects on viral transcription and replication, Nef has been shown to down-regulate expression of cell surface CD4 (15, 16, 18). It is also reported to inhibit the antigen-mediated induction of interleukin-2 (IL-2) mRNA (29). Early studies with Nef indicated G-protein activity (19); however, our studies along with several others indicate that this was most probably due to bacterial contaminants (4, 5, 23, 36). Considered together, these properties suggest that Nef may interfere with signalling pathways, other than G protein, involved in T-cell activation and proliferation.

Recently, our laboratory reported that direct insertion of the 27-kDa isoform of Nef (Nef 27), produced in *Escherichia coli* by translation from the first start codon of HIV-1 *nef* clone pNL4-3, into cells causes the down-regulation of cell surface CD4 and IL-2 receptor (IL-2R) expression without affecting a variety of other cell surface molecules (18). In contrast, Nef 25,

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a second Nef isoform of 25 kDa produced in *E. coli* by translation from the second start codon and missing the first 19 amino acid residues from the N terminus, had no effect on surface CD4 and IL-2R (18). These data show that Nef decreases expression of two cell surface receptors crucial for antigen recognition of major histocompatibility complex class II antigens and for cell proliferation.

Full characterization of the function of Nef and investigation into its precise target(s) within the cell requires identification of cellular proteins with which Nef interacts. To address this issue, Nef 27 and Nef 25 were produced as C-terminal fusion proteins with glutathione *S*-transferase in *E. coli* and used as affinity reagents. In this study, we report that Nef 27 interacts specifically with at least seven cellular proteins, four of which have been identified as the Src family kinase p56^{lck}, the cell surface receptor CD4, the antioncogene product p53, and the mitogen-activated protein kinase pp44^{mapk/erk1}. When highly purified Nef 27 was introduced directly into peripheral blood mononuclear cells (PBMC), it inhibited IL-2-induced cell proliferation and activation of the Src family kinase p56^{lck} in response to IL-2 or phorbol 12-myristate 13-acetate (PMA) stimulation. Further to this, treatment of human T-cell leukemia virus type I-transformed MT-2 cells with Nef 27 reduced expression of the nucleoprotein c-Myb, which is required for transition of cells from G₀/G₁ of the cell cycle (56). In contrast to the protein interactions and biological effects observed with Nef 27, Nef 25 was shown to interact with only three cellular proteins, one of which was identified as p56^{lck}, albeit more weakly. Furthermore, treatment of PBMC with Nef 25 had less or no effect on IL-2-induced proliferation or activation of p56^{lck} in response to IL-2 or PMA stimulation.

MATERIALS AND METHODS

Cells. MT-2 cells (kindly provided by Y. Hinuma, Institute for Virus Research, Kyoto University, Kyoto, Japan) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 µg of glutamine per ml, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (CSL, Melbourne, Australia) in a 5% CO₂ atmosphere at 37°C. PBMC were isolated from HIV-1-seronegative volunteers by centrifugation on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient (40). PBMC were activated with phytohemagglutinin (PHA; 10 µg/10⁶ cells) for up to 72 h at 37°C and cultured in supplemented RPMI 1640 medium. PBMC were prepared for electroporation either before or after stimulation with PHA or PMA. For protein precipitation experiments, PBMC were harvested from culture after stimulation with PHA (as described above).

Expression of recombinant HIV-1 Nef in *E. coli*. The large-scale expression in *E. coli* and purification of HIV-1 Nef 27 and Nef 25 (pNL4.3 clone) either alone or as GST-Nef 27 and GST-Nef 25 fusion proteins have been described elsewhere (4).

Electroporation of *E. coli*-derived HIV-1 Nef 27 or Nef 25 into MT-2 cells and PBMC. Electroporation of recombinant Nef 27 or Nef 25 (corresponding to HIV-1 Nef derived from the pNL4.3 clone) into MT-2 cells and PBMC has been described elsewhere (18). Cells (5 × 10⁵) were electroporated with 1.0 µM Nef 27 or Nef 25. Control cells included those that were electroporated with bovine serum albumin (BSA) at an equivalent concentration (1 µM BSA) or without protein (mock electroporated).

Antibodies specifically recognizing HIV-1 Nef. An antibody specific for HIV-1 Nef was raised in sheep immunized with a peptide corresponding to the predicted amino acid residues 15 to 27 (AVRERMRRAEPAA) of Nef encoded by the HIV-1 clone pNL4.3 as described previously (18). This antibody is termed sheep anti-Nef₍₁₅₋₂₇₎.

Detection of Nef in electroporated cells by indirect immunofluorescence. Immediately after electroporation of MT-2 cells or PBMC, the cells were washed in phosphate-buffered saline (PBS) and prepared for detection of incorporated Nef by indirect immunofluorescence staining as described previously (18).

Precipitation and identification of cellular proteins binding to GST-Nef 27 or GST-Nef 25 fusion protein. MT-2 cells or PHA-activated PBMC (3 × 10⁷ cells per sample) were washed twice in PBS, resuspended in 300 µl of lysis buffer (0.5% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 50 mM NaCl, 25 mM Tris-HCl, 10 mM EDTA, 5 mM benzamide-HCl, 10 mM phenylmethylsulfonyl fluoride in *n*-propanol, 0.01% [wt/vol] sodium azide [pH 8.0]) and incubated on ice for 5 min. After this incubation period, the cell lysate was centrifuged at 12,000 × *g* for 10 min and the cytoplasmic extract was recovered. The cytoplasmic fraction was then precleared by incubation with 50 µl of a 50% slurry (in Tris-buffered saline [TBS; pH 7.4]) of glutathione-Sepharose 4B (Pharmacia) at 4°C for 30 min. After centrifugation at 12,000 × *g* for 2 min, the

supernatant was recovered and divided into four 1.5-ml tubes (Eppendorf, Hamburg, Germany), and GST (1 µM per sample), GST-Nef 27 (1 µM per sample), GST-Nef 25 (1 µM per sample), or TBS was added. The cell supernatants and added proteins were then incubated together overnight at 4°C. On the following day, 50 µl of a 50% slurry of glutathione-Sepharose beads was added to each tube, and the tubes were incubated with continuous agitation for 30 min at room temperature. Each suspension was then cooled on ice for 15 min before the Sepharose was pelleted by centrifugation for 2 min at 12,000 × *g*. The glutathione-Sepharose beads were washed three times with 1.5 ml of ice-cold TBS containing 0.05% (vol/vol) Nonidet P-40. Bound proteins were eluted by incubation of the Sepharose beads with 20 µl of 10 mM glutathione for 10 min at room temperature. After centrifugation, the supernatant was removed and stored, and the elution step was repeated twice. Aliquots (20 µl) of the eluted material were then electrophoresed on a 13% polyacrylamide gel, and the separated material was either detected by silver staining or transferred to Hybond C-Super nitrocellulose (Amersham International, Amersham, United Kingdom) and immunoblotted with antibodies (monoclonal or polyclonal) reactive with ZAP 70, Raf-1, v-H-Ras, c-Yes, c-Jun, p53, p34^{cdc2}, pp44^{mapk/erk1}, p59^{lyn}, p56^{lck} (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.); several rabbit polyclonal antibodies reactive with p56^{lck} (anti-p56^{lck}; Santa Cruz) (polyclonal antibody 377, which was raised against a peptide corresponding to amino acid residues 476 to 509 of human p56^{lck}, polyclonal antibody 278, which was raised against a peptide corresponding to amino acid residues 387 to 399 of human p56^{lck}, and polyclonal antibody 277, which was raised against a peptide corresponding to amino acid residues 39 to 64 of human p56^{lck} [a gift from T. Potter and A. Clayton, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo.] [6, 8, 58]); or polyclonal antibodies reactive with CD4 (hyperimmune rabbit serum to recombinant soluble CD4; a gift from J. Culp, Smith Kline Beecham).

For Western immunoblotting, the nitrocellulose filters were preincubated with 5% (wt/vol) BLOTTO for 2 h at room temperature and then reacted with each of the antibodies (anti-p53, anti-p34^{cdc2}, anti-pp44^{mapk/erk1}, anti-ZAP 70, anti-p56^{lyn}, anti-p59^{lyn}, anti-p56^{lck}, anti-c-Jun, anti-Raf-1, anti-c-Yes, and anti-v-H-Ras [diluted 1:500]; Santa Cruz), anti-p56^{lck} antibodies 377, 278, and 277 [diluted 1:1,000], and anti-CD4 [diluted 1:1,000] overnight at room temperature. As controls, normal rabbit or rat serum or isotype-matched control mouse antibodies were also included. After this incubation period, the filters were washed extensively in PBS-0.05% (wt/vol) Tween 20 and incubated with donkey anti-rabbit immunoglobulin (Ig) conjugated to biotin, sheep anti-rat Ig conjugated to biotin, or sheep anti-mouse Ig conjugated to biotin (diluted 1:1,000; Amersham) for 1 h at room temperature. After extensive washing as described above, the filters were incubated with streptavidin-conjugated horseradish peroxidase (diluted 1:1,000; Amersham) for 1 h at room temperature. After further washing, the filters were developed with 1,4-dichloronaphthol as the substrate.

The specificity of GST-Nef interactions with cellular proteins was confirmed by competitive inhibition studies with recombinant Nef 27 and Nef 25. For the competition studies, recombinant Nef 27 (at 1, 2, or 4 µM per sample) or Nef 25 (at 1, 2, or 4 µM per sample) was incubated with the glutathione-Sepharose-purified MT-2 cell or PBMC cytoplasmic extracts at the same time as GST-Nef 27 or GST-Nef 25, respectively.

Proliferation of PBMC in response to IL-2. Growth factor-dependent DNA synthesis was determined by measurement of [³H]thymidine incorporation into cellular DNA as previously described (1). Briefly, PHA-activated PBMC electroporated with Nef 27 or Nef 25 or mock electroporated as described above were incubated in supplemented RPMI 1640 medium for 24 h at a concentration of 10⁶/ml and then stimulated with IL-2 (Boehringer, Mannheim, Germany) at 10, 30, or 100 IU/ml (triplicate samples of 10⁶ cells per sample). After 24 h in culture, the cell cultures were counted, the percentage viable cells was estimated by using trypan blue exclusion as a marker (all cultures contained more than 95% viable cells), cells were pulsed with [³H]thymidine (5 µCi/ml) for 16 h, and incorporation of radioactivity into DNA was measured by liquid scintillation counting. Each sample was assayed in triplicate. The values presented are the means ± standard deviations for three experiments.

Activation of p56^{lck} by IL-2 or PMA stimulation in HIV-1 Nef-treated PBMC. Human PBMC were isolated from normal adults by Ficoll-Paque density centrifugation (40). Cells were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum in the presence of PHA (10 µg/10⁶ cells) for 3 days. The PHA-activated PBMC were then washed twice in PBS and incubated in supplemented RPMI 1640 medium for 24 h to deplete any low-level production of IL-2. Following this incubation, the PHA-activated PBMC were electroporated with Nef 27 or Nef 25 or mock electroporated as described above. After culturing in medium for a further 24 h, the cells were harvested, washed twice in PBS, and then incubated with 1,000 IU of recombinant IL-2 (Boehringer) for 0, 15, and 30 min. After the incubation, cells were lysed and analyzed for the presence of phosphorylated forms of p56^{lck} by immunoblotting with anti-p56^{lck} antibodies 377, 277 and 278 (Santa Cruz) as described above. For activation of PBMC by PMA, cells were electroporated with Nef 27, Nef 25, or BSA or mock electroporated as described above. After washing in PBS, the cells were stimulated with PMA (20 ng/10⁶ cells) for 4 h at 37°C. The cells were then harvested and prepared for immunoblotting with anti-p56^{lck} as described above.

Detection of c-myc levels in Nef-treated cells. MT-2 cells, harvested during mid-exponential growth phase, were washed twice in PBS and electroporated with recombinant Nef 27, Nef 25, or BSA or mock electroporated as described above. After electroporation, the cells were washed twice in PBS, resuspended at

FIG. 1. Transfer of Nef 27 and Nef 25 into cells by electroporation. MT-2 cells (A, C, and E) and PBMC (B, D, and F), either electroporated with Nef 27 (A and B) or Nef 25 (C and D) or mock electroporated (E and F), were washed, fixed, permeabilized, and reacted with sheep anti-Nef₍₁₅₋₂₇₎ followed by fluorescein isothiocyanate-conjugated donkey anti-sheep Ig as described previously (14). Cells were photographed at a magnification of $\times 800$, using an epifluorescence microscope with excitation at 488 nm.

a concentration of 10^6 cells per ml in supplemented medium, and incubated for 24 h at 37°C. Following the incubation period, the cells were washed in PBS again and resuspended in lysis buffer at a concentration of 10^8 cells per ml, and cellular debris was removed by centrifugation. Cell lysate corresponding to 2×10^6 cells was then electrophoresed in a 13% polyacrylamide gel, transferred to Hybond C-Super nitrocellulose, and immunoblotted with anti-c-Myb diluted 1:1,000 (anti-c-Myb antibodies were kindly provided by R. Ramsay, Ludwig Cancer Research Institute, Melbourne, Australia) (42) or anti-c-Jun diluted 1:500 (Santa Cruz) as described above.

RESULTS

Detection of Nef in electroporated cells by indirect immunofluorescence. Cells electroporated with Nef 27 or Nef 25 were examined for uptake of Nef by indirect immunofluorescence. Examination of permeabilized MT-2 cells and PBMC electroporated with Nef 27 by using anti-Nef₍₁₅₋₂₇₎ showed that virtually the entire population of cells contained Nef 27 after electroporation (Fig. 1). Similarly, virtually 100% of MT-2 cells and PBMC electroporated with Nef 25 showed intense fluorescence when reacted with anti-Nef₍₁₅₋₂₇₎, indicating that the entire cell populations contained Nef 25 after electroporation (Fig. 1). Reaction of mock-electroporated cells with anti-Nef₍₁₅₋₂₇₎ produced only background staining, confirming the specificity of the antibodies for Nef (Fig. 1). The pattern of fluorescence that was observed after reaction of anti-Nef₍₁₅₋₂₇₎ with Nef 27- or Nef 25-containing cells showed that both proteins were predominantly localized at the plasma membrane (Fig. 1).

Staining of both permeabilized and nonpermeabilized cells

with a monoclonal antibody that recognizes an epitope in the C-terminal region of Nef (AE6) and that gave an excellent low-background staining under both conditions confirmed that the protein was inside the cell and not merely adhering to the cell membrane (data not shown). Incubation of the cells with Nef 27 or Nef 25, without electroporation, and subsequent reaction with anti-Nef₍₁₅₋₂₇₎ produced only low-level background fluorescence, again confirming that Nef had entered the cell (data not shown).

Effect of Nef on IL-2- and PMA-dependent activation of PBMC. The proliferative response of PBMC to IL-2 and mitogenic lectins requires the interaction of IL-2 with the high-affinity (α , β , and γ chains) or intermediate-affinity (β and γ chains) IL-2R complex (44, 48, 52, 53). Electroporation of Nef 27 into PHA-activated PBMC reduced the proliferative response of these cells to subsequent IL-2 exposure by up to 71% (Nef 27, 30 IU of IL-2 per ml; Table 1). Nef 25-treated cells also showed some reduction in proliferative response to IL-2 (up to 35%, 10 IU of IL-2 per ml; Table 1). The IL-2 proliferative response of PHA-activated PBMC which were mock electroporated or electroporated with BSA increased in parallel with the concentration of IL-2 (Table 1). The level of thymidine incorporation observed both before and after stimulation concurs with levels previously reported and is probably related to low-level induction of IL-2 by stimulation of PBMC with PHA (32). Thymidine incorporation was markedly less in

TABLE 1. Effects of Nef 27 and Nef 25 on IL-2-induced proliferation of PBMC^a

Experimental condition	³ H]thymidine incorporation (cpm/ml) following stimulation with the indicated concentration of IL-2 (IU/ml)			
	0	10	30	100
Untreated	18,445	35,440	79,510	105,430
Mock	15,120	34,325	80,038	103,868
BSA	17,889	34,788	82,335	105,676
Nef 25	10,973	22,660	58,432	86,937
Nef 27	8,895	17,945	23,938	53,441

^a PHA-activated PBMC electroporated with Nef 27, Nef 25, or BSA or mock electroporated were incubated (10^6 cells per ml) in supplemented RPMI 1640 medium for 24 h as described in Materials and Methods. The cells were then stimulated with IL-2 at 0, 10, 30, or 100 IU/ml for 24 h. Following this incubation, [³H]thymidine was added and the cells were incubated for a further 16 h. Values are means for three experiments; the standard deviation was always less than $\pm 10\%$.

Nef 27-treated cells than in with mock-treated and BSA-treated cells even before stimulation with IL-2 and again may be related to reduced responsiveness of Nef-treated cells to IL-2 induced by PHA treatment. Nef 25 also decreased the level of thymidine incorporation, albeit less markedly.

Both tyrosine and serine/threonine protein kinases are involved in IL-2-dependent proliferative signals (7, 33). Indeed, p56^{lck} tyrosine kinase activity rapidly increases following IL-2 addition and undergoes subsequent IL-2-dependent serine/threonine phosphorylation (21, 22). Modification by phosphorylation retards electrophoretic mobility of p56^{lck} (22). Addition of IL-2 to mock-electroporated PBMC resulted in reduced electrophoretic mobility of p56^{lck}, consistent with phosphorylation of p56^{lck} (Fig. 2, tracks 1 to 3). The more slowly migrating species of p56^{lck} was found to comigrate with the altered-mobility species of p56^{lck} induced after PMA treatment of PBMC (data not shown). While mock-electroporated, IL-2-stimulated PBMC showed normal conversion of most p56^{lck} to its p60 isoform, phosphorylation of p56^{lck} did not occur in detectable amounts in IL-2-treated PBMC electroporated with Nef 27 (Fig. 2, tracks 4 to 6). Interestingly, in contrast to the effect of Nef 27, Nef 25 did not significantly inhibit phosphorylation of p56^{lck} in response to IL-2 treatment, as most p56^{lck} was phosphorylated to its p60^{lck} form in Nef 25-containing PBMC after treatment with IL-2 (Fig. 2, tracks 7 to 9).

The phorbol ester PMA induces the activation of protein kinase C (PKC) in T cells (59). A number of cellular proteins including p56^{lck} are phosphorylated on serine/threonine residues as a result of PKC activation (30, 59). Treatment of

kDa

FIG. 2. Western immunoblotting of Nef-treated PBMC lysate with anti-p56^{lck} before and after IL-2 stimulation. PHA-activated PBMC mock electroporated (tracks 1 to 3) or electroporated with Nef 27 (tracks 4 to 6) or Nef 25 (tracks 7 to 9) were treated with IL-2 (1,000 U) for 0 min (tracks 1, 4, and 7), 15 min (tracks 2, 5, and 8), or 30 min (tracks 3, 6, and 9), lysed, and electrophoresed in a 13% polyacrylamide gel as described in Materials and Methods. After transfer to Hybond C-Super nitrocellulose, the filters were reacted with anti-p56^{lck} as described in Materials and Methods.

kDa

FIG. 3. Western immunoblotting of Nef-treated PBMC lysates with anti-p56^{lck} before and after PMA stimulation. PBMC mock electroporated (tracks 1 and 2) or electroporated with Nef 27 (tracks 3 and 4) or Nef 25 (tracks 5 and 6) were treated with PMA (20 ng/ml; tracks 1, 3, and 5) or incubated in medium alone (tracks 2, 4, and 6) for 2 h at 37°C as described in Materials and Methods. Cell lysates were electrophoresed, transferred to Hybond C-Super nitrocellulose, and subsequently probed with anti-p56^{lck} as described in Materials and Methods.

PBMC with Nef 27 also inhibited the phosphorylation of p56^{lck} in response to PMA treatment (Fig. 3). No inhibitory effect was observed in PBMC treated with Nef 25 (Fig. 3).

The effects of Nef on IL-2- and PMA-mediated responses can be considered as specific and not due to nonspecific cytotoxicity. Cells electroporated with either Nef 27 or Nef 25 remain close to 100% viable ($>95\%$ viable) for at least 96 h postelectroporation. Previously, we described that the down-regulatory effect of Nef 27 on cell surface receptor expression was specific for CD4 and IL-2R, since expression of other cell surface receptors such as transferrin receptor, CD7, and CD2 was not affected (18). Collectively, these factors indicate the effects of Nef to be specific for CD4 and IL-2R expression, p56^{lck} phosphorylation and activation, and cell proliferation among those functions studied.

Expression of c-myb in Nef-treated MT-2 cells. The nuclear oncoprotein Myb is believed to be an important regulator of cell growth and differentiation in hematopoietic cells and is required for transition of cells from the G₀/G₁ phase of the cell cycle to early S phase (56). Furthermore, optimal expression of the CD4 gene requires a Myb transcription factor (49). Introduction of Nef 27 and also Nef 25 into MT-2 cells reduced the levels of c-myb expressed, while levels of c-myb were not affected in mock-electroporated cells (Fig. 4A). The effect of Nef 25 on the level of c-myb expression was not as marked as that

A
kDa B
kDa

FIG. 4. Levels of nucleoproteins c-Myb and c-Jun in Nef-treated MT-2 cells as detected by Western immunoblotting. MT-2 cells electroporated with Nef 27 (track 3) or Nef 25 (track 2) or mock electroporated (track 1) were lysed, electrophoresed, transferred to Hybond C-Super nitrocellulose, and reacted with anti-c-Myb (A) or anti-c-Jun (B) as described in Materials and Methods.

FIG. 5. Detection of cellular proteins which interact with Nef 27 or Nef 25 by silver staining. Cellular protein precipitates prepared from MT-2 cell (A and C) and PHA-activated PBMC (B and D) lysates after reaction with TBS (track 3), GST (track 4), GST-Nef 27 (tracks 5, A and B), or GST-Nef 25 (tracks 5, C and D) were glutathione-Sepharose affinity purified, electrophoresed, and silver stained as described in Materials and Methods. Track 1 represents GST-Nef 27 (2 μ g; A and C) or GST-Nef 25 (2 μ g; B and D), and track 2 represents GST protein (2 μ g) in each case.

of Nef 27. In contrast to the effect of Nef on *c-myc* expression, treatment of cells with either Nef 25 or Nef 27 did not affect the levels of *c-jun* expression (Fig. 4B), indicating that effect on nucleoprotein expression was specific for *c-myc*.

Binding of cellular proteins to Nef. Incubation of GST-Nef 27 fusion protein with lysates prepared from MT-2 cells coprecipitated seven cellular proteins, approximately 24, 27, 32, 36, 44, 50, and 56 kDa in size (Fig. 5A). When GST-Nef 27 was used as an affinity reagent to identify proteins from PHA-activated PBMC interacting with Nef, eight proteins of 27, 28, 30, 32, 36, 56, 60, and 75 kDa were present (Fig. 5B). Some differences in molecular weights of the protein species interacting with GST-Nef 27 were observed between MT-2 cells and PBMC. This may represent differences in proteins able to interact with Nef but more likely may reflect the different amounts and types of proteins present in different cell types. In contrast to those proteins precipitated with GST-Nef 27, when GST-Nef 25 was used as the binding antigen, only proteins of approximately 27, 28, and 56 kDa were precipitated from MT-2 cells and from PHA-activated PBMC lysates, as identified by silver staining (Fig. 5C and D, respectively). No detectable proteins were precipitated when GST was incubated with any of the precleared lysates. Similarly, no detectable proteins were precipitated by interaction with the glutathione-Sepharose beads alone (TBS control), indicating that the proteins precipitated with GST-Nef 27 and GST-Nef 25 formed specific interactions with the Nef proteins (Fig. 5). Binding of these cellular proteins to Nef was specific as addition of one- to fourfold molar excess of recombinant Nef 27 or Nef 25 inhibited the binding of GST-Nef 27 or GST-Nef 25 to all MT-2 cell- and PBMC-derived proteins in a dose-dependent fashion (data not shown).

Identification of cellular proteins binding to GST-Nef 27 and GST-Nef 25 by use of specific antibodies. Immunoblotting with various antibodies to p53, p34^{cdc2}, ZAP 70, pp44^{map/erk1}, p56^{lyn}, p59^{hyn}, c-Jun, c-Yes, Raf-1, v-H-Ras, p56^{lck} and CD4 was used to identify the cellular proteins which coprecipitated with Nef. These antibodies were chosen because of the potential link of Nef with disturbance of T-cell receptor- and IL-2R-mediated signal transduction pathways identified from our earlier studies, which showed that Nef altered CD4 and IL-2R expression, and results of other reports which described that Nef could inhibit NF κ B transcriptional activity (18, 37). In particular, anti-CD4 was chosen since Nef modulates its surface expression (15, 16, 18), and antibodies which recognize members of the Src family of proteins (p56^{lck}, p56^{lyn} and p59^{hyn}) were chosen because these proteins are known to modulate signalling from and associate with IL-2R (25) and, in the case of p56^{lck}, also because of its association with CD4 (6, 45, 58). The antibody to ZAP 70 was chosen because of this proteins involvement in T-cell receptor-mediated cell activation (60); antibodies to c-Yes, Raf-1, v-H-Ras, and pp44^{map/erk1} were chosen since these proteins are involved in signal transduction (7, 35, 55, 57); antibodies to c-Jun, p53, and p34^{cdc2} (3, 26, 34) were chosen because of the involvement of these proteins in cell cycle control. Reaction of anti-p56^{lck} with MT-2 cell- and PHA-activated PBMC-derived proteins which coprecipitated with GST-Nef 27 detected the presence of p56^{lck} (Fig. 6A, tracks 3 and 6, respectively). p56^{lck} most likely represents the protein identified by silver staining as 56 kDa. The 60-kDa phosphorylated isoform of p56^{lck} present in lysates of PHA-activated PBMC was also shown to coprecipitate with Nef 27 (Fig. 6A, track 6). The 60-kDa isoform of p56^{lck} was not identified in cel-

FIG. 6. Identification of cellular proteins which interact with Nef 27 and Nef 25 by immunoblotting. Shown are immunoblots of cellular protein precipitates prepared from MT-2 cell (tracks 1 to 3) or PHA-activated PBMC (tracks 4 to 6) lysates after reaction with GST (tracks 1 and 4), GST-Nef 25 (tracks 2 and 5), or GST-Nef 27 (tracks 3 and 6) followed by glutathione-Sepharose affinity purification. Anti-p56^{lck} antibody 377 (A), anti-CD4 (B), anti-p53 (C), and anti-p44^{mapk/erk1} (D) were used as the primary antibodies.

lular proteins precipitated from MT-2 cell lysates, possibly because of lower amounts of p56^{lck} in MT-2 cells than in PBMC (data not shown). The specificity of the reaction with anti-p56^{lck} was verified by using four separate antibodies which recognize p56^{lck} and was most clearly shown by anti-p56^{lck} (Santa Cruz) which was raised against a peptide corresponding to amino acids 476 to 505 within the carboxyl-terminal domain of human p56^{lck} and reacts with human and mouse p56^{lck} but lacks detectable cross-reactivity with other members of the Src protein kinase family.

Immunoblotting of GST-Nef 27 protein precipitates prepared from both MT-2 cells and PBMC showed the presence of CD4 as a band of approximately 55 kDa (Fig. 6B, tracks 3 and 6, respectively). MT-2 cell lysates precipitated with GST-Nef 27 contained both p53 and anti-pp44^{mapk/erk1}, as bands of approximately 53 and 44 kDa, respectively (Fig. 6C and 6D, respectively, tracks 3). p53 was also present in the GST-Nef 27 precipitate prepared from PHA-activated PBMC (Fig. 6C, track 6); however, pp44^{mapk/erk1} could not be detected, possibly because of lower levels present in PBMC (data not shown). The presence of p53 in GST-Nef 27 protein precipitates prepared from either MT-2 cells or PHA-activated PBMC was clearly evident only after specific immunoblotting. Detection of a 53-kDa protein corresponding to p53 by silver staining was most likely masked by GST-Nef 27, p56^{lck}, and CD4. Reaction of the GST-Nef 27 cellular protein precipitates with normal rabbit serum or matched-isotype antibody controls confirmed

all antibody reactions to be specific (data not shown). Similarly, when cellular material precipitated with GST alone was probed with the specific antibodies, no positive reactions were observed (Fig. 6A to C, tracks 1 and 4 in each case; Fig. 6D, track 1). Interaction of GST-Nef 27 with p53, CD4, p56^{lck}, and pp44^{mapk/erk1} could be inhibited in a dose-dependent manner by competition with Nef 27 (complete inhibition of protein interaction could be achieved by using a fourfold molar excess of protein), confirming the specific interaction of Nef 27 with these proteins (Fig. 7A, C, and E to I). Reaction of GST-Nef 27 precipitates prepared from PHA-activated PBMC or MT-2 cells showed the absence of detectable levels of p56^{lyn}, c-Yes, c-Jun, v-H-Ras, Raf-1, or p59^{lyn}, ZAP 70, and p34^{cdc2} (data not shown).

In contrast to findings with Nef 27, when cellular proteins precipitated from MT-2 cells or PBMC with GST-Nef 25 were reacted with an antibody to p53, p34^{cdc2}, ZAP 70, pp44^{mapk/erk1}, p56^{lyn}, p56^{lck}, p59^{lyn}, or CD4 in immunoblotting, only p56^{lck} was present in detectable levels (Fig. 6A to C [tracks 2 and 5] and Fig. 6D [track 2] show immunoblots probed with anti-p56^{lck}, anti-CD4, anti-p53, and anti-pp44^{mapk/erk1}). However, the reactivity of Nef 25 with p56^{lck} was less than that of Nef 27. As described above, cellular material precipitated with GST alone did not react with anti-p56^{lck}, while the interaction of p56^{lck} with GST-Nef 25 could be inhibited by Nef 25, confirming the specific interaction of Nef 25 with p56^{lck} (Fig. 7B and D).

FIG. 7. Inhibition of interaction of cellular proteins which interact with the GST-Nef fusion proteins by Nef 27 and Nef 25. Shown are immunoblots of cellular protein precipitates prepared from MT-2 cell (A, B, E, G, and I) or PHA-activated PBMC (C, D, F, and H) lysates after reaction with GST (track 1 in each case), GST-Nef 25 (tracks 2, B and D), or GST-Nef 27 (tracks 2, A, C, and E to I) followed by glutathione-Sepharose affinity purification. In competition studies, lysates from MT-2 cells (A, B, E, G, and I) or PHA-activated PBMC (C, D, F, and H) were preincubated with Nef 27 (A, C, and E to I) or Nef 25 (B and D) at onefold (track 3), twofold (track 4), or fourfold (track 5) molar excess before reaction with GST-Nef 27 or GST-Nef 25, respectively, and subsequent glutathione-Sepharose affinity purification. Anti-p56^{lck} antibody 377 (A to D), anti-CD4 (E and F), anti-p53 (G and H), and anti-pp44^{mapk/erk1} (I) were used as the primary antibodies.

DISCUSSION

For the first time, we show that Nef 27 inhibits the proliferative response of PHA-activated PBMC to IL-2. Treatment of PBMC with Nef 27 inhibited the IL-2- and PMA-induced phosphorylation of the Src family kinase p56^{lck}, known to be associated with the early events of T-cell activation and signaling, and also markedly reduced expression of nucleoprotein c-Myb, which is required for transition of cells from the G₀/G₁ to S phase of the cell cycle (56). Our studies have also identified several cellular proteins which specifically interact with Nef 27, four of which were identified as p56^{lck}, CD4, p53, and pp44^{mapk/erk1} by using a panel of 12 antibodies to various signal transduction-cell cycle proteins. All of these proteins are intimately linked in cell activation and cycling.

Recently we reported that Nef protein down-regulates the expression of the IL-2R α chain (18). The receptor for IL-2 comprises three distinct polypeptide chains, α , β , and γ . The α chain binds IL-2 with low affinity, and the β and γ chains afford binding of intermediate affinity; together, the three receptor chains constitute the high-affinity receptor (53). Introduction of Nef 27 protein into PHA-activated PBMC reduced the proliferative response of these cells to IL-2, possibly as a consequence of its down-regulatory effect on IL-2R α -chain expression. However, since we have previously reported that introduction of Nef 25 protein into PHA-activated PBMC had no obvious effect on the expression of the IL-2R α chain (18),

we hypothesize that Nef prevents proper IL-2 signalling further down the IL-2 signalling pathway. It is known that IL-2-induced proliferative signals can be delivered by the intermediate-affinity as well as the high-affinity IL-2R. Nef may perturb components of the IL-2R complex other than the α chain. Alternatively, Nef may interfere with proteins associated with IL-2 R which are critical in mediating signal transduction following IL-2 stimulation. Thus, we investigated the effect of Nef on activation of p56^{lck}, a member of the Src family of non-receptor tyrosine kinases, known to associate with the β subunit of the IL-2R complex and to play a critical role in mediating IL-2 signal transduction (13, 22).

IL-2 stimulation of PBMC provokes both augmentation of p56^{lck} activity and increased posttranslational (serine/threonine phosphorylation) modification (22). The conversion of the kinase to its p60 isoform during the IL-2 response may be used as a surrogate marker of p56^{lck} activation (33). Treatment of PHA-activated PBMC with Nef 27 completely inhibited detectable IL-2-induced phosphorylation and hence activation of p56^{lck}, which may, in part, cause the observed reduced responsiveness to IL-2 stimulation. Inhibition of p56^{lck} phosphorylation may be a direct result of interaction of Nef with p56^{lck} as described herein or may indicate inhibition of serine/threonine kinases such as PKC (30, 59) or the mitogen-activated protein kinase family, known to phosphorylate p56^{lck} (55). Indeed, stimulation of Nef 27-treated PBMC with PMA, a known ac-

tivator of PKC, failed to induce conversion of p56^{lck} to its phosphorylated p60 isoform.

Introduction of Nef protein into MT-2 cells was also shown to affect the levels of the proto-oncogene *c-myc*. Several studies provide evidence that *c-myc* gene function is specifically required for transition through late G₁ or early S phase of the cell cycle (56). The inhibitory effects of Nef on high-affinity IL-2R, which is a classical marker of T-cell competence, and expression of *c-myc*, which represents one of the last gene products to appear before DNA synthesis, provide evidence that Nef interferes with both early and late stages of lymphocyte activation.

Identification of p56^{lck}, CD4, pp44^{mapk/erk1}, and p53 as proteins which interact with Nef 27 highlights a role for Nef in modulating cell signalling pathways. The exact nature of the protein interactions is currently being investigated in our laboratory. The role of p56^{lck} in signal transduction in response to IL-2 stimulation has been discussed above. CD4 plays an important role in activation of mature T lymphocytes, acting in synergy with the antigen-specific T-cell receptor for induction of transmembrane signals that result in lymphokine production (45). The role of CD4 in assisting antigen-specific signal transduction may in part be mediated by p56^{lck}, since p56^{lck}, which associates with CD4 via residues in its unique N-terminal region, is known to interact with and phosphorylate chains of the T-cell receptor (45). Hence, in addition to the inhibitory effect on IL-2 signalling, Nef may interfere with antigen-mediated activation through the T-cell receptor as suggested by others (37, 50). The association of Nef with pp44^{mapk/erk1} and p53 may also be of significance to the function of Nef since pp44^{mapk/erk1} belongs to a family of protein serine/threonine kinases that play important roles in intracellular signalling. Specifically, pp44^{mapk/erk1} may be involved in the phosphorylation of p56^{lck} to its p60 isoform (55), while p53 can function as an inhibitor of cell cycle progression, presumably acting at some stage before the G₁-to-S transition, although action by other means cannot be excluded (61).

The present study highlights fundamental differences in activity between the 27- and 25-kDa forms HIV-1 Nef. In contrast to Nef 27, Nef 25 only modestly affects cell proliferation (18% inhibition, compared with 50% for Nef 27, at 100 IU of IL-2 per ml; Table 1). Nef 25 did not bind CD4, pp44^{mapk/erk1}, or p53, exhibited reduced binding capacity for p56^{lck}, and had no effect on phosphorylation of this protein (Fig. 2 and 6) compared with Nef 27. A modest reduction in *c-myc* expression was observed in Nef 25-treated cells (Fig. 4). These results indicate that although Nef 25 has some functions similar to those of Nef 27, they are reduced in number and potency.

Point mutation studies have shown that myristoylation of the N-terminal glycine residue of Nef is important for its association with the cytoskeletal matrix (38). However, close inspection of the data reveals only a two- to fourfold loss of localization when the myristoylation acceptor glycine codon was replaced by alanine (38). Our results herein indicate that despite the absence of a myristoyl group on mature Nef protein, immediate plasma membrane localization still occurred when the protein was introduced into cells by electroporation. A recent review implicates that localization of myristoylated protein at the plasma membrane depends not only on myristoylation but also on the N-terminal sequence of the protein (43). It is well known that myristoylated proteins can localize to the cytosol as well as various intracellular membranes (43). Nef 27 possesses N-terminal sequences (2GKWSKSSVIGWPAVR ERMRR21) that may assist in its plasma membrane localization, while Nef 25 contains the last two residues of this sequence (43). The reduced activity exhibited by Nef 25 in comparison with Nef 27 may be due in part to the lack of the first 19 amino acids. Whether these are responsible for precise membrane localization or protein conformation or represent a func-

tional domain of the molecule remains to be determined. In support of Nef 25 retaining some activity, if we increase the amount of Nef 25 used to treat cells by sixfold, then we do observe a modest effect on CD4 and IL-2R surface expression (17). Finally, although both Nef 27 and Nef 25 are grossly located at the plasma membrane, they may be associated with different complexes of host cell proteins, influencing their final functions.

Using GST-Nef fusion proteins as affinity reagents, we have developed a highly sensitive system for identifying cellular proteins which interact with Nef. In our hands, the GST-Nef fusion protein-based precipitation system has proven more sensitive and reliable than conventional immunoprecipitation techniques, probably reflecting the relatively low amounts of Nef protein in HIV-1-infected cells (17). Studies using a *nef* gene product expressed as an N-terminal fusion protein with GST (Nef-GST) describe multiple cellular proteins, derived from Jurkat cells, which interact with Nef (20). These proteins of approximately 280, 97, 75, 55/57, 35, 32, 28, and 26 kDa show extensive molecular weight overlap with those identified in this study, despite the different Nef fusion protein constructs used and a radiolabel method for identification. Other studies which used a hybrid CD8-Nef fusion protein which was constitutively expressed in T-cell lines or chronically infected T-cell lines with HIV-1SF2 in conjunction with traditional immunoprecipitation methods showed Nef to be associated with at least three proteins: 62- and 72-kDa proteins and a cellular serine kinase of unknown size (46). These were identified only in a sensitive *in vitro* kinase assay. Constitutive expression of Nef either by transfection of a CD8-Nef expression plasmid or by chronic infection may have selected for cells which can overcome the effects of Nef, and hence the proteins interacting with Nef may be different.

Our data indicate that Nef inhibits normal T-cell function and signalling probably as a consequence of its interaction with specific intracellular targets. Divergent findings for the effect of Nef on HIV-1 replication have been reported (2, 9, 28, 31, 39, 51, 54). Subtle effects on replication have been observed *in vitro*, while in contrast, simian immunodeficiency virus *nef* was shown to be essential for maintenance of high virus load and progression to disease in macaques (24). Our data would suggest that by decreasing T-cell activation, Nef acts as a positive factor for the HIV-1 life cycle by inhibiting virus-induced apoptosis, postulated as one mechanism of HIV cell killing and other cell activation-linked processes (27). By controlling the degree of cell activation, Nef protein may be allowing the maximum production of infectious progeny, an observation supported from *in vitro* studies with *nef*-deleted virus replication (10, 31, 51).

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