Human Immunodeficiency Virus Type 1 Nef Binds to Tumor Suppressor p53 and Protects Cells against p53-Mediated Apoptosis

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The *nef* gene product of human immunodeficiency virus type 1 (HIV-1) is important for the induction of AIDS, and key to its function is its ability to manipulate T-cell function by targeting cellular signal transduction proteins. We reported that Nef coprecipitates a multiprotein complex from cells which contains tumor suppressor protein p53. We now show that Nef interacts directly with p53. Binding assays showed that an N-terminal, 57-residue fragment of Nef (Nef 1-57) contains the p53-binding domain. Nef also interacted with p53 during HIV-1 infection in vitro. As p53 plays a critical role in the regulation of apoptosis, we hypothesized that Nef may alter this process. Nef inhibited UV light-induced, p53-dependent apoptosis in MOLT-4 cells, with Nef 1-57 being as effective as its full-length counterpart. The inhibition by Nef of p53 apoptotic function is most likely due to its observed ability to decrease p53 protein half-life and, consequently, p53 DNA binding activity and transcriptional activation. These data show that HIV-1 Nef may augment HIV replication by prolonging the viability of infected cells by blocking p53-mediated apoptosis.

Infection with human immunodeficiency virus type 1 (HIV-1) causes progressive loss in CD4 lymphocyte numbers and function, resulting in the immunodeficiency associated with AIDS (11). The mechanisms by which CD4 lymphocytes are depleted during HIV-1 infection are not well understood, but the HIV-1 *nef* gene is a key determinant of accelerated CD4 lymphocyte depletion in vivo (7, 26, 28).

The *nef* gene of HIV-1 encodes a 25- to 30-kDa myristoylated protein which is produced early during infection by translation from several singly and multiply spliced mRNA species (45, 49). In infected cells Nef localizes at the plasma membrane and preferentially associates with the cytoskeleton, but it is also found in the cytoplasm, at the nuclear membrane, and in the nucleus (27, 32, 40, 41, 43). Three key functions of Nef, which are potentially interrelated, may explain its contribution to disease. The first function is a highly conserved ability to down-regulate cell surface CD4 and major histocompatibility complex class 1 molecules, the second is the ability to augment virus infectivity, and the third is the ability to modulate multiple cellular signaling pathways in both CD4 lymphocytes and macrophages (for a review see reference 20). Nef protein regulation of T-cell activation and associated pathways most likely directly influences both virion infectivity and expression of the cellular receptors involved in T-cell activation, including CD4.

The effect of Nef on T-cell signaling is complex. This fact is highlighted by the number of cellular signal transduction elements, including CD4, NAK, Raf-1, mitogen-activated protein kinase (MAPK), and tumor suppressor protein p53, which have been shown to bind to Nef (6, 20, 24). Targeting of these

proteins by Nef may represent an integrated approach by which this HIV protein controls both cellular and viral components of the virus life cycle to augment virus production. However the basis for and effect of most of these interactions have not been fully characterized.

Localization of Nef in the cytoplasm and nucleus suggests that it may control signal transduction events other than those which occur immediately at the plasma membrane. Nuclear localization of Nef in HIV-1-infected cells directly suggests that it may act as a nuclear regulatory factor. We previously reported that a glutathione *S*-transferase (GST)–Nef fusion protein coprecipitated tumor suppressor protein p53, among other cellular proteins, from CD4⁺ T cells (17). The ability of Nef to bind to p53 and the overlapping subcellular distribution of Nef and p53 within the cytoplasm and nucleus suggest that Nef may influence p53 function (27, 30, 40). p53 has been shown to regulate HIV-1 gene expression by suppressing transcriptional activation of the long terminal repeat (LTR) (9, 47). However, as p53 can cause cell growth arrest and induce apoptosis and since a number of virus-encoded proteins influence these activities (for a review see reference 55), p53 association with Nef may also modulate these activities in a manner that enhances HIV-1 replication.

In this study we show that the Nef protein of HIV-1, via its N terminus (amino acid residues 1 to 57), interacts directly with p53. This interaction results in the destabilization of p53, thereby decreasing its proapoptotic, transcriptional, and DNA binding activities. These data support an important role for Nef in controlling p53 protein function during the HIV-1 life cycle.

MATERIALS AND METHODS

Cell lines and primary cells. The human leukemia CD4-expressing T-cell line MOLT-4 (wild-type p53 [15]), human T-cell leukemia virus type 1 (HTLV-1)-

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transformed T-cell line MT-2 [wild-type p53 [44]], and erythroleukemic cell line K562 (p53 null [8]) were maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 0.2 mM glutamine, 50 U of penicillin/ml, and 50 µg of streptomycin/ml. Human osteosarcoma Saos-2 cells (p53 null [48]) and 293T cells [inactive [48]] were grown in Dulbecco's modified Eagle's medium supplemented as described above.

Expression of recombinant HIV-1 Nef or p53 proteins in *Escherichia coli*. The full-length Nef protein, corresponding to the *nef* sequence from HIV-1 molecular clone NL4-3, fragments of Nef corresponding to amino acid residues 1 to 57 (Nef 1-57), 1 to 79 (Nef 1-79), and 20 to 206 (Nef 20-206), simian immunodeficiency virus (SIV) Nef corresponding to the *nef* sequence from SIV molecular clone mac239 (SIVmac239), and p53 protein were expressed and purified either alone or as GST fusion proteins as described previously (2).

For the expression of GST-Nef fusion proteins corresponding to the *nef* genes of primary HIV-1 strains isolated from two patients with well-documented HIV-1 infection and disease progression, peripheral blood mononuclear cells were isolated from blood by Ficoll-Paque density centrifugation and lysed as previously described (7). Cell lysates were then subjected to a first-round PCR amplification using oligonucleotide primers SK-68 and CI-6. The PCR products were then used in a second-round amplification using primers specific to the *nef* LTR region. All primer sequences and PCR conditions have been previously reported (7). The *nef* LTR PCR products were then cloned into pGEM-7z(+) (Promega, Madison, Wis.) and sequenced as previously described. The consensus *nef* sequence was generated by computer analysis, and the clone most homologous to this consensus was selected for cloning into pGEX 4T-1 (Pharmacia, Uppsala, Sweden) with the following oligonucleotide primers: 5' primer 5'-GC GGAATTCGGTGGCAAGTGGTCAAAATG-3' and 3' primer 5'-ATAAGAA TGC GGCGCTCAGTTCCTGTAGAACTCCGGGTGCAAC-3' for patient C23-4 and 5' primer 5'-GCTCCGGATCCATGGGTGGCAAGTGGTCAAAA CG-3' and 3' primer 5'-ATAAGAATGCGGCCGCTCAGTTCCTGTAGTAC TCCGGATGCAGC-3' for patient C42. These were used in conjunction with the PCR protocol previously described (19). These primers contain *Bam*HI (5' primers) and *Not*I (3' primers) restriction sites which were utilized to clone the sequences into pGEX 4T-1 as previously described. The fusion proteins were then expressed as described previously (2, 19).

Constructs. Proviral plasmids pNL4-3 and pNL4-3-nef-stop were previously described (16). WWP-Luc, which contains a p53-binding element upstream of a WAF 1 promoter-luciferase reporter gene construct, and DM-Luc, which represents WWP-Luc with the p53-binding element deleted were kindly provided by B. Vogelstein (Oncology Center, The Johns Hopkins University School of Medicine, Baltimore, Md.) (10). pCEP4SN, which represents human wild-type p53 cloned into pCEP4 (cytomegalovirus [CMV]-driven expression plasmid) was also kindly donated by B. Vogelstein. pCMV-Luc contains the *Renilla* firefly luciferase gene under the control of the intermediate-early gene promoter of CMV. The *nef* gene of HIV-1 molecular clone NL4-3 was also placed under the control of the intermediate-early gene promoter of CMV in pEGFP-N1 (Clontech, Palo Alto, Calif.). pEGFP-N1 was also used as a marker of transfection efficiency.

Infection of CD4⁺ T-cell lines with HIV-1. MT-2 and MOLT-4 cells were infected with equivalent inocula (100 ng of p24) of HIV-1 NL4-3 or HIV-1 NL4-3-nef-stop. Virus-infected cells and as a control mock-infected cells were incubated for up to 6 days, and the levels of virus production were determined by assay of cell-free reverse transcriptase activity (46).

Detection of direct interaction of Nef with p53 using coprecipitation and enzyme-linked immunosorbent assays. (i) **Coprecipitation assay.** Purified p53 (1.4 µM) was diluted in phosphate-buffered saline (PBS) containing 0.05% (vol/vol) NP-40 and incubated with GST-Nef (1.4 µM) or as a control GST alone (1.4 µM) for 2 h at room temperature. Potential GST-Nef and p53 complexes were precipitated using glutathione-Sepharose (Pharmacia) as previously described (17). Eluted GST-Nef-p53 complexes were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose (Schleicher & Schuell). Antibodies against p53 (murine monoclonal antibody; Santa Cruz Biotechnology, Santa Cruz, Calif.; diluted 1:1,000) were used for immunochemical detection using ECL (Amersham, Little Chalfont, United Kingdom).

To ascertain that the Nef protein encoded by primary HIV-1 strains and SIV also interact with p53, the *nef* genes from HIV-1 strains isolated from two HIV-1-infected individuals and from SIVmac239 were expressed as GST-Nef fusion proteins and included in the coprecipitation assays. Purified p53 (1.4 µM) was diluted in PBS containing 0.05% (vol/vol) NP-40 and incubated with GST-Nef (1.4 µM) or as a control GST alone (1.4 µM) as described above. Potential complexes of GST-Nef with p53 were precipitated and detected as described above.

The specificity of the GST-Nef-p53 interaction was verified by competitive

inhibition studies using a purified recombinant Nef protein. For the competition studies, p53 (1.4 µM) was incubated with a recombinant Nef protein at a 0- to 30-fold molar excess prior to the addition of GST-Nef (1.4 µM) and the potential complexes of GST-Nef with p53 were precipitated and detected as described above.

(ii) **Direct enzyme-linked immunosorbent assay.** Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 50 µl of purified recombinant Nef (200 nM) or as a control GST at the same concentration and diluted in PBS for 2 h at 37°C. After the remaining sites of the wells were blocked with 150 µl of 1% (wt/vol) gelatin dissolved in PBS, 50 µl of purified p53 (0 to 100 nM) or PBS alone was added to the wells and the wells were incubated for 2 h at 37°C. Anti-p53 (Santa Cruz Biotechnology) or an isotype-matched control monoclonal antibody diluted in PBS to the same immunoglobulin concentration was then added to each well (50 µl/well), and the wells were incubated at 37°C for 1.5 h. The binding of the antibodies to the immobilized protein complexes was detected as previously described (18).

Identification of amino acid residues of Nef which interact with p53. Protein fragments corresponding to amino acid residues 20 to 206 and 1 to 57 of Nef, their full-length counterpart, and GST were used to coat (200 nM, diluted in PBS) the wells of 96-well plates for 2 h at 37°C. By methodology described above, purified p53 (0 to 100 nM) or PBS was added to the wells in 50-µl aliquots, and the wells were incubated for 2 h at 37°C. Binding of p53 to the Nef fragments was detected as described above.

To assess whether the N-terminal fragment of Nef corresponding to amino acids 1 to 57 bound to p53 and another Nef-interacting protein, Lck, purified Nef 1-57 (1.0 µM) was incubated with cellular extracts derived from MOLT-4 cells. The extracts were prepared by incubation of the cells with lysis buffer, centrifugation, and preclearance with protein G-Sepharose as previously described (17). Purified recombinant Nef 1-57 was then incubated with the extracts for 2 h at 4°C. Next a monoclonal antibody reactive with Nef 1-57 or a matched isotype control antibody was incubated with the extracts for 4 h at 4°C. Following this incubation, protein G-Sepharose was added to precipitate antibody-protein complexes, and the presence of p53 or Lck in the complexes was determined by SDS-PAGE followed by electrophoretic transfer to nitrocellulose (Schleicher & Schuell) and Western blotting using anti-p53 (Santa Cruz Biotechnology) or antibodies specific for Lck (Santa Cruz Biotechnology) and ECL (Amersham).

Coimmunoprecipitation of Nef with p53 from HIV-1-infected cells. For immunoprecipitation of Nef from HIV-1-infected cells, total-cell extracts from virus-infected (HIV-1 NL4-3 or HIV-1 NL4-3-nef-stop) and mock-infected MOLT-4 cells and MT-2 cells were prepared as described above. Equal amounts of protein from precleared lysates were then incubated with anti-Nef monoclonal antibody AE6 (this reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health [from James Hoxie]) or a matched isotype control for 4 h at 4°C. Following this incubation, protein G-Sepharose-antibody-protein complexes were prepared. To detect p53, the relevant immunoprecipitates were immunoblotted for p53 as described above.

Transfection of cells. For transient transfection of Saos-2 and K562 cells, a calcium phosphate DNA precipitation methodology was used (16). The transfection efficiency was determined by cotransfection of an enhanced green fluorescent protein (EGFP) reporter construct (16). Twenty-four hours posttransfection cells were harvested from culture and cells which were successfully transfected as identified by the expression of EGFP were sorted and recovered by flow cytometry. The recovered cells were then manipulated according to the experimental requirements.

MOLT-4 cells were transiently transfected with the appropriate vectors by electroporation as described previously (19). The transfection efficiency was determined by cotransfection of an EGFP reporter construct (16). At 48 h posttransfection the cells were harvested from culture, and those successfully transfected, as determined by the expression of EGFP, were sorted and recovered by flow cytometry.

Electroporation of Nef protein into MOLT-4 and Saos-2 cells. MOLT-4 cells were electroporated with various full-length or truncated Nef proteins corresponding to the *nef* gene from HIV-1 NL4-3 or HIV-1 C42 (0 to 1.0 µM) or GST (0 to 1.0 µM) or were subjected to mock electroporation (without protein) as described previously (21). Immediately after electroporation the uptake of Nef or GST proteins by the MOLT-4 cells was detected with antibodies specific for Nef or GST by indirect immunofluorescence procedures (21). The cells were then returned to culture for 24 h. For the transfected Saos-2 cells, cells which were transfected (as identified by EGFP expression) were electroporated with increasing concentrations of full-length Nef protein (0 to 1.0 µM) or GST (0 to 1.0 µM) or were mock electroporated. Routinely, Nef and GST proteins were taken up by at least 95% of cells. The cells were then returned to culture for up

FIG. 1. Nef binds to p53. (a) A GST-Nef fusion protein specifically coprecipitates p53. Purified recombinant GST-Nef (lane 1) and GST (lane 6) alone were incubated with purified recombinant p53, affinity purified with glutathione-Sepharose beads, electrophoresed, and transferred to nitrocellulose. Purified p53 was electrophoresed and transferred to nitrocellulose as a control (lane 7). Nitrocellulose membranes were then reacted with anti-p53 in Western blotting. For competition of the GST-Nef-p53 interaction by purified Nef protein, p53 was incubated with purified Nef protein at 0.3- (lane 2), 3- (lane 3), 10- (lane 4), and 30-fold (lane 5) molar excess before reaction with GST-Nef and processing as described above. (b) Nef from primary HIV-1 isolates and SIV bind to p53. Purified recombinant GST-Nef corresponding to the *nef* genes from HIV-1 NL4-3 (lane 2), HIV-1 C42 (lane 3), HIV-1 C23-4 (lane 4), and SIVmac239 (lane 5) or GST (lane 1) was incubated with purified recombinant p53, affinity purified with glutathione-Sepharose beads, electrophoresed, and transferred to nitrocellulose. Nitrocellulose membranes were then reacted with anti-p53 in Western blotting as described above. (c) Direct interaction between p53 and Nef as detected by a solid-phase binding assay. Solutions of purified recombinant Nef and GST at 200 nM were used to coat the wells of 96-well polystyrene microtiter plates. After being coated and blocked with gelatin the wells were incubated with increas-

to 12 h, after which time they were harvested for inclusion in the luciferase assay (see below).

Exposure of Nef-treated MOLT-4 cells to UV irradiation. Following transfection for Nef and EGFP expression in the case of MOLT-4 cells and for Nef, p53, and EGFP expression in the case of K562 cells, the positively transfected cells were isolated by flow cytometry. These sorted cells and those electroporated directly with Nef proteins were resuspended at a concentration of 10^7 cells/ml in RPMI 1640 supplemented with 0.1% (vol/vol) FCS and exposed to a UV light source (4 J/m^2) for approximately 30 s. Cells were then cultured in RPMI 1640 supplemented with 0.1% (vol/vol) FCS for 12 to 16 h, after which time they were analyzed for the induction of apoptosis by DNA laddering and annexin V-fluorescein isothiocyanate (FITC) or annexin V-Cy5 staining (Boehringer GmbH, Mannheim, Germany). MOLT-4 cells infected with HIV-1 NL4-3 or HIV-1 NL4-3-nef-stop or mock infected were exposed to UV light as described above. The HIV-1-positive cells were analyzed for the induction of apoptosis by dual staining with polyclonal human sera from HIV-1-infected individuals and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) with FITC (TUNEL-FITC) (Boehringer GmbH).

Annexin-V staining, DNA ladder assay, and TUNEL staining. Induction of apoptosis in response to UV treatment was detected by a DNA ladder assay as previously described (54). For MOLT-4 cells which had been electroporated with mature Nef proteins, detection of apoptosis at the single-cell level was performed by propidium iodide and annexin V-FITC staining in conjunction with flow-cytometric analysis. For MOLT-4 cells which had been cotransfected for Nef and EGFP expression and for K562 cells which had been cotransfected for Nef, p53, and EGFP expression, detection of apoptosis at the single-cell level was done by propidium iodide and annexin V-Cy5 staining. For staining with annexin V-FITC or annexin V-Cy5 the cells were reacted with binding buffer containing the annexin V conjugate and propidium iodide according to the manufacturer's instructions (Boehringer GmbH) and analyzed by flow cytometry.

For detection of apoptotic HIV-1-infected cells using TUNEL cells were fixed with 3.5% paraformaldehyde for 30 min at room temperature. Following fixation the cells were reacted with pooled human polyclonal sera from HIV-1-infected individuals or control sera from uninfected individuals for 1 h at room temperature. After this the cells were reacted with anti-human immunoglobulin conjugated to biotin (Amersham) for 1 h on ice followed by reaction with streptavidin conjugated to phycoerythrin (Becton Dickinson). The cells were then permeabilized with 0.1% (vol/vol) Triton X-100 and then reacted with reagents from the In Situ Cell Fluorescein Death Detection kit (Boehringer GmbH) according to the manufacturer's instructions and analyzed by flow cytometry or fluorescence microscopy.

Luciferase assay. Transfected and electroporated Saos-2 cells were harvested from culture and lysed in Reporter lysis buffer (Promega). Lysates were added to luciferase assay substrate (Promega), and luminescence was measured.

Nuclear extraction. Nuclear extracts were prepared from 10^8 MOLT-4 cells essentially as previously described (1).

EMSA. Double-stranded oligonucleotides having the p53 consensus DNA binding sequences 5'-CCGAGACATGCCTAGACATGCCT-3' and 5'-CCGAGGCGATGTCTAGGCATGTCT-3' were commercially obtained and radio-labeled by end filling with [α - ^{32}P]dCTP and DNA polymerase I. For the electrophoretic mobility shift assay (EMSA), binding reactions were carried out as previously described (53). Purified recombinant Nef protein and as a control GST were added to test samples in equal amounts (1.0 to 3.0 μM). After a further incubation at 4°C for 40 min, reaction mixtures were electrophoresed on a 4% polyacrylamide nondenaturing gel at 4°C in $0.5\times$ Tris-borate-EDTA.

Measurement of p53, Nef, and WAF 1 levels by immunoblotting. The lysates derived from the transfected and electroporated Saos-2 cells, samples prepared for EMSA, lysates prepared from UV-stimulated MOLT-4 cells, and lysates prepared from HIV-1-infected MOLT-4 cells were measured for p53, Nef, and WAF 1 and combinations of these three molecules and actin levels with anti-p53 (Santa Cruz Biotechnology), anti-Nef (21), anti-WAF 1 (Santa Cruz Biotechnology), or anti-Oct-1 and actin (as a protein loading control; Santa Cruz Biotechnology) by immunoblotting as described above.

p53 protein stability. MOLT-4 cells were electroporated with full-length Nef, Nef 20-206, and Nef 1-57 derived from the *nef* gene of HIV-1 NL4-3, with full-length Nef derived from HIV-1 C42, or, as a control, with GST or were mock

ing amounts of recombinant p53 (0 to 100 nM). The binding of Nef was detected with anti-p53. The results represent values obtained after subtraction of values for the control antibody from those obtained when the p53-specific antibody was incorporated into the assay.

FIG. 2. p53 binds to the N-terminal region of Nef. Solutions of purified recombinant protein fragments corresponding to amino acid residues 20 to 206, 1 to 79, and 1 to 57, full-length Nef, and GST at 200 nM were used to coat wells of a 96-well polystyrene microtiter plate and incubated with increasing amounts of recombinant p53. Binding of p53 to the Nef fragments was detected using anti-p53. The results represent values obtained after subtraction of values for the control antibody from those obtained when the p53-specific antibody was incorporated into the assay.

electroporated as described above. For pulse-chase experiments the cells were incubated in methionine-free medium for 30 min and labeled with 500 μ Ci of [35 S]methionine/ml for 30 min. Cells were then washed and transferred to culture medium supplemented with cold 4 mM methionine. The cells were harvested from culture at various intervals, and protein extracts were prepared. Proteins were immunoprecipitated with anti-p53 (Pab421; Oncogene) and analyzed by SDS-PAGE. For further assessment of the Nef effect on p53 levels, MOLT-4 cells were electroporated with full-length Nef derived from HIV-1 NL4-3 or HIV-1 C42, Nef 20-206, or GST or were mock electroporated as described above and p53 levels were measured by Western blotting as described above.

p53 mRNA quantitation. p53 mRNA levels from MOLT-4 cells electroporated with Nef proteins or GST and exposed to UV light as described above were assessed by Northern hybridization 12 to 16 h after treatment (14). p53 mammalian expression plasmid pCEP4SN was used in PCR amplification using synthetic oligonucleotide primers corresponding to the nucleotide sequence of p53 (22). As a control, oligonucleotide primers corresponding to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were also constructed to amplify a GAPDH sequence. The resulting DNA products were then radiolabeled with [α - 32 P] dCTP by random priming with a commercially available kit (Boehringer GmbH) for use as probes in Northern hybridization as previously described (58).

RESULTS

Nef binds to p53 via its N-terminal domain. We have previously shown that Nef can specifically bind a cellular multi-protein complex containing wild-type p53 (17). To demonstrate that Nef can directly interact with p53, purified recombinant Nef and p53 were used in *in vitro* binding assays. A GST-Nef fusion protein, corresponding to the *nef* gene of HIV-1 molecular clone NL4-3 specifically coprecipitated purified recombinant p53 (Fig. 1a). Competition of the GST-Nef and p53 interaction with increasing concentrations of purified recombinant Nef protein alone showed that the interaction between the two proteins occurred approximately on a 1:1 molar basis. Competition of the interaction with 0.3-, 3-, 10- and 30-fold molar excesses of Nef reduced the amount of p53 recovered with the GST-Nef coprecipitate by approximately 25, 75, 90, and 92%, respectively, as determined by densitometry (Fig. 1a). GST-Nef fusion proteins corresponding to the *nef* genes of HIV-1 primary isolates C42 and C23-4 and SIV-mac239 were also able to specifically coprecipitate p53, highlighting the conserved nature in HIV-1 and SIV of this interaction (Fig. 1b). p53 also bound specifically to immobilized

purified recombinant Nef in a concentration-dependent manner (Fig. 1c).

A series of Nef protein fragments were used to map the p53-binding site on Nef. p53 interacted with full-length Nef and with fragments Nef 1-79 and Nef 1-57 but not with Nef 20-206 (Fig. 2). Inclusion into the solid-phase binding assay of a fragment of Nef corresponding to amino acid residues 1 to 20 did not support binding to p53 (data not shown). Thus, the N-terminal 57-amino-acid region of Nef is the predominant region involved in the Nef-p53 interaction. The failure of amino acids 1 to 20 and 20 to 206 of Nef to support binding to p53 suggests that the conformation of the protein or the junction of these two regions may be important for the interaction.

The relevance of the Nef interaction with p53 to the infection process was also confirmed as immunoprecipitation of Nef from HIV-1-infected CD4⁺ lymphoblastoid cells (MT-2 cells [data not shown] and MOLT-4 cells [Fig. 3]) coprecipitated p53. p53 was specifically coimmunoprecipitated from HIV-1 NL4-3-infected cells with anti-Nef antibodies (Fig. 3, lane 7) but not from mock-infected or HIV-1 NL4-3-nef-stop-infected cells (Fig. 3, lanes 5 and 6, respectively). Immunoblotting for p53 of the extracts used for immunoprecipitation is included as a control (Fig. 3, lane 1). Lane 1 is not intended to be used for comparison with the amount of p53 which coprecipitates with Nef (Fig. 3, lane 7) as the amount of the extract used for coprecipitation of Nef and p53 is significantly greater (approximately 30-fold) than the amount used in the immunoblotting of p53. Given that both proteins can localize to the nucleus, it is highly probable that the association between Nef and p53 occurred prior to lysis of the cells (40, 41, 60).

Nef protects MOLT-4 cells against UV-induced apoptosis. We tested whether Nef binding to p53 affected p53-mediated apoptosis induced by UV irradiation (a p53-dependent event [25, 31, 33]). We compared the susceptibilities of Nef-treated and untreated MOLT-4 cells which express wild-type p53 to UV irradiation. Mock-electroporated and GST-electroporated MOLT-4 cells exposed to lethal doses of UV light undergo rapid p53-dependent apoptosis as determined by annexin V staining and DNA fragmentation (Table 1 and Fig. 4). However MOLT-4 cells electroporated with full-length Nef protein resisted UV-induced cell death, as evidenced by only back-

FIG. 3. Nef associates with p53 during *in vitro* HIV-1 infection of CD4⁺ T cells. Cell lysates were prepared from mock-infected and HIV-1 NL4-3- and HIV-1 NL4-3-nef-stop-infected MOLT-4 cells and were reacted with either anti-Nef to immunoprecipitate (IP) Nef and its associated cellular proteins (lanes 5 to 7) or a matched isotype control (lanes 2 to 4). The immunoprecipitates were then separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-p53. Total-cell lysate from mock-infected MOLT-4 cells was also immunoblotted with anti-p53 as a control (lane 1).

TABLE 1. Nef protects cells against UV-induced apoptosis^a

Construct	Mean % of apoptotic cells ± SEM	
	+ UV	No UV
None (mock electroporated)	64.2 ± 2.8	14 ± 2.3
GST	61 ± 3.6	10.8 ± 3.1
Nef _{HIV-1 NL4-3}	11.7 ± 2.1	10.4 ± 3.5
Nef _{HIV-1 C42}	13.5 ± 3.5	8.6 ± 2.4
Control plasmid	21.5 ± 3.5	11.4 ± 3.2
p53	51 ± 5.5	15.6 ± 3.2
p53 + Nef _{HIV-1 NL4-3}	18.4 ± 4.2	17.5 ± 4.0

^a MOLT-4 cells which had been electroporated with full-length Nef corresponding to the *nef* gene from HIV-1 NL4-3 or HIV-1 C42 or, as controls, GST or no protein (mock-electroporated) were exposed to UV light and returned to culture. Cells induced to undergo apoptosis were measured by annexin V-FITC and propidium iodide staining in conjunction with flow-cytometric analysis (10,000 events). K562 cells which had been cotransfected with expression plasmids for EGFP, Nef, and p53 or control plasmids were sorted by flow cytometry for EGFP-positive cells, exposed to UV light, and then returned to culture. Cells induced to undergo apoptosis were measured by annexin V-Cy5 and propidium iodide staining in conjunction with flow-cytometric analysis (10,000 events).

ground annexin V-FITC staining and no detectable fragmentation of DNA (Table 1 and Fig. 4). Inhibition by Nef of UV-induced apoptosis in MOLT-4 cells was dose dependent, highlighting the specific effect of Nef (data not shown). Nef expressed in MOLT-4 cells from plasmid DNA also prevented UV-induced apoptosis, and the degree of protection afforded by Nef expressed during transient transfection was comparable to that given by the electroporated Nef protein (data not shown). Inclusion of Nef derived from the HIV-1 C42 into the UV-induced apoptosis assay clearly showed that this primary isolate-derived Nef protein also protected the MOLT-4 cells from UV-induced apoptosis (Table 1). These data suggest that this function of Nef is conserved in HIV-1.

The specific involvement of p53 in the apoptotic process induced by UV stimulation was confirmed by analyzing the sensitivity to UV stimulation of K562 cells transfected for p53 expression relative to that of K562 cells transfected with a control plasmid. K562 cells transfected with a control plasmid

FIG. 4. Nef protects cells against UV-induced apoptosis. MOLT-4 cells which had been electroporated with purified recombinant full-length Nef or GST or which had been mock electroporated were either exposed to a lethal dose of UV light (+) for approximately 30 s or were not exposed (–) and then returned to culture for 12 h. Hirt DNA was extracted from each of the samples and analyzed by gel electrophoresis for the presence of fragmented DNA. Hirt DNA extracted from untreated MOLT-4 cells was also analyzed (U/T).

TABLE 2. The N terminus of Nef protects cells against UV-induced apoptosis^a

Nef construct	Mean % of apoptotic cells ± SEM	
	+ UV	No UV
None (mock electroporated)	61.4 ± 5.8	6.8 ± 1.8
Nef (1-206)	8.0 ± 1.2	6.0 ± 1.7
Nef (1-57)	8.1 ± 2.3	6.6 ± 1.8
Nef (20-206)	63.2 ± 6.2	7.7 ± 1.6

^a MOLT-4 cells were electroporated with recombinant full-length Nef protein (Nef 1-206) or fragments of Nef protein corresponding to amino acid residues 20 to 206 (Nef 20-206) or 1 to 57 (Nef 1-57) or were mock electroporated as a control. Following electroporation with the various proteins the cells were exposed to UV light and returned to culture. Cells induced to undergo apoptosis were measured by annexin V-FITC and propidium iodide staining in conjunction with flow-cytometric analysis (10,000 events).

were relatively resistant to the apoptosis-inducing effects of UV stimulation at this level of exposure (Table 1). This finding is consistent with other reports (36). In contrast, K562 cells expressing p53 displayed substantially increased sensitivity to UV radiation (Table 1). Cotransfection of K562 cells for Nef and p53 expression showed that Nef was able to inhibit p53-induced apoptosis in response to UV stimulation (Table 1).

Next we compared each of the Nef fragments used in the Nef-p53 binding studies for their abilities to protect MOLT-4 cells against UV-induced apoptosis. Nef proteins which bound p53 in binding assays (full-length Nef and Nef 1-57) protected against apoptosis, while cells electroporated with a Nef protein which did not bind p53, Nef 20-206, were not protected at all (Table 2).

To ascertain that the antiapoptotic effect of the N-terminal fragment of Nef was due to its interaction with p53 and not with Lck (3), which has been proposed to bind to the N-terminal region of Nef, we assessed whether Nef 1-57 bound Lck. Immunoprecipitation of Nef 1-57 from cell lysates containing both p53 and Lck and the sequential immunoblotting of the immunoprecipitates showed that Nef 1-57 bound p53 but not Lck (data not shown).

Mechanisms by which Nef inactivates p53. (i) **Nef reduces p53 DNA binding activity, p53 transcriptional activation, and p53 levels.** The mechanism by which p53 induces apoptosis is still unclear; however, studies suggest that p53-induced apoptosis may be mediated by p53 transcriptional activation of genes such as *bax* (5, 38). Thus, we investigated whether the ability of Nef to bind to p53 affected p53 DNA binding activity and transcriptional activation. By EMSA, multiple specific complexes comprising an endogenous wild-type human p53 from MOLT-4 cells and a DNA oligonucleotide containing a consensus-binding site for p53 were formed (Fig. 5a, lane 2). The complexes may represent dimeric, tetrameric, and oligomeric forms of p53 (10, 56). Their formation can be inhibited by using a nonlabeled form of the oligonucleotide containing a consensus binding site for p53 (Fig. 5a, lane 3) but not by nonspecific oligonucleotides (Fig. 5a, lane 4). Inclusion of a purified recombinant Nef protein in the assay system resulted in decreased interaction of p53 with its DNA binding sequence in a concentration-dependent manner (Fig. 5a, lanes 6 to 8), while GST had no effect (Fig. 5a, lane 5).

As a consequence of inhibiting p53 DNA binding activity

FIG. 5. Nef reduces binding of p53 to DNA, p53-mediated transcriptional activation, and p53 protein levels. (a) Nef reduces p53 binding to DNA. Nuclear extracts prepared from MOLT-4 cells were preincubated with increasing amounts of purified recombinant Nef or GST proteins before reaction with [α - 32 P]dATP-labeled human p53 consensus site DNA binding or random control oligonucleotides. DNA binding events were analyzed by EMSA. Samples include nuclear extracts incubated with either the labeled consensus p53 binding site oligonucleotide (lane 2), the labeled consensus p53 binding site oligonucleotide and an excess of the same unlabeled "cold" oligonucleotide (lane 3), the labeled consensus p53 binding site oligonucleotide and excess unlabeled random-sequence oligonucleotides (lane 4), increasing amounts of purified Nef protein (lanes 6 to 8), or GST (lane 5) prior to incubation with the labeled consensus p53 binding site oligonucleotide. Lane 1, labeled oligonucleotide only. Nef added to nuclear extracts prior to formation of p53-DNA complexes inhibited specifically the formation of these complexes, while addition of GST had no effect. (b) Nef reduces p53-mediated transcriptional activation. A luciferase reporter under the control of a p53-dependent WAF 1 promoter (WWP-Luc) was introduced into Saos-2 cells with or without p53 as indicated. Twenty-four hours after transfection the cells were electroporated with increasing amounts of purified recombinant Nef 1-206 (0.1 to 3.0 μ M), Nef 20-206 (3.0 μ M), Nef 1-57 (3.0 μ M), or GST (3.0 μ M). Mock-electroporated cells were used as a control. The luciferase activities measured represent average values of three experiments. (c) Nef reduces p53 protein levels. The nuclear extracts prepared from the MOLT-4 cells incubated either alone (no treatment; lane 1) or with GST (3.0 μ M; lane 2) or increasing amounts of purified Nef protein (1.0 to 3.0 μ M; lanes 3 to 6) were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose filters were immunoblotted with anti-p53 (top), anti-Oct-1 (middle), or anti-Nef (bottom). (d) Lysates were prepared from Saos-2 cells which had been transfected for p53 and WWP-Luc expression and subsequently electroporated with Nef 1-206 (0.1 to 3.0 μ M; lanes 3 to 6), Nef 20-206 (3.0 μ M; lane 2), Nef 1-57 (3.0 μ M; lane 7), or GST (3.0 μ M; lane 1). The lysates were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose filters were immunoblotted with anti-p53 (top) or antiactin (middle). Lysates derived from cells electroporated with Nef 1-206 (3.0 μ M), GST (3.0 μ M), Nef 20-206 (3.0 μ M), or Nef 1-57 (3.0 μ M) were also immunoblotted with anti-Nef (bottom). (e) WAF 1 levels are reduced during HIV-1 infection of MOLT-4 cells with HIV-1 NL4-3. MOLT-4 cells were infected with HIV-1 NL4-3 (lane 2) or HIV-1 NL4-3-nef-stop (lane 3) or were mock infected (lane 1). After a determination by immunofluorescence staining of HIV antigens that similar numbers of cells (~50% of the population) were infected by each virus, whole-cell extracts were prepared. The extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-WAF 1 (top) or antiactin (bottom).

FIG. 6. (a) Nef inhibits the induction of p53 protein in response to UV treatment. MOLT-4 cells which had been electroporated with recombinant full-length Nef 1-206 (lanes 1 and 6), Nef 20-206 (lanes 3 and 8), or Nef 1-57 (lanes 2 and 7) and controls electroporated with GST (lanes 4 and 9) or mock electroporated (lanes 5 and 10) were exposed to UV light (lanes 6 to 10), and 12 h later whole-cell extracts were prepared. Following normalization of total protein levels the extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-p53 (top), antiactin (middle), or anti-Nef (bottom). (b) Nef does not alter p53 mRNA levels. MOLT-4 cells which had been electroporated with 3.0 μ M recombinant full-length Nef 1-206 (lanes 4 and 5), Nef 1-57 (lanes 8 and 9), or GST (lanes 6 and 7) or which, as a control, had been mock electroporated (lanes 2 and 3) were exposed to UV light and then returned to culture for 12 h. After this time period total RNA was isolated and incubated with a 32 P-labeled probe specific for p53 mRNA (top) or GAPDH mRNA (bottom) using Northern hybridization. Total RNA samples from untreated MOLT-4 cells (lanes 1 and 10) and MT-2 cells (lane 11) were included as controls. Total RNA samples from K562 cells (lane 12), which are p53 null, were included as a negative control.

Nef may also inhibit p53-dependent transcriptional activity. To address this, Saos-2 cells transfected with wild-type p53 and a p53-responsive luciferase reporter plasmid containing the WAF 1 promoter (WWP-Luc) were treated with the full-length Nef protein to determine the effect of Nef on p53-mediated transcriptional activation. Nef dramatically and specifically inhibited p53-driven luciferase expression in a concentration-dependent manner (Fig. 5b, lanes 3 to 6), while addition of GST had no effect (Fig. 5b, lane 7). The inhibitory effect of Nef fragments on p53-mediated transcriptional activation appeared to directly correlate with the ability of Nef to bind p53, as Nef 1-57 inhibited p53-driven luciferase expression but Nef 20-206 had no effect (Fig. 5b, lanes 8 and 9). Nef did not have a general effect on transcription, as the levels of CMV promoter-driven luciferase from CMV-Luc were not altered (data not shown).

Extracts from experiments outlined in Fig. 5a and b were used in immunoblot analysis to determine the effect of Nef on p53 levels and whether an alteration in levels could explain the observed effects of Nef on p53 binding to DNA and p53-mediated transcription. Addition of full-length Nef to MOLT-4 and Saos-2 cells resulted in a dose-dependent decrease in endogenous (MOLT-4 cells; Fig. 5c, top, lanes 3 to 6) and exogenous p53 protein levels (Saos-2 cells; Fig. 5d, top, lanes 1 to 4). The binding of Nef to p53 correlated with a decrease in p53 protein, as Nef 1-57 also caused a decrease in p53 protein levels (Fig. 5d, top, lane 7) while GST alone and Nef 20-206 had no effect (Fig. 5d, top, lanes 5 and 6). The lack of effect of Nef 20-206 on p53 levels is not due to the instability of this protein, as similar levels of Nef 20-206, Nef 1-206, and Nef 1-57 were detected in the extracts after immunoblotting (Fig. 5d, bottom). Nef had no effect on Oct-1 levels (Fig. 5c, middle) or actin levels (Fig. 5d, middle). Thus, the binding of Nef to p53 correlates with a decrease in the steady-state levels of p53 and a subsequent loss of p53 DNA binding activity and

p53-mediated transcriptional activation and may explain these effects.

To confirm the effect of Nef on p53-dependent transcription events during HIV-1 infection in vitro, the levels of WAF 1 expressed during infection of MOLT-4 cells with HIV-1 NL4-3 or HIV-1 NL4-3-nef-stop relative to those expressed by mock infected cells were assessed. Assessment of the numbers of cells infected by HIV-1 NL4-3 and HIV-1 NL4-3-nef-stop by immunofluorescence studies using antisera specific to HIV-1 proteins showed that conservatively 50% of each of the populations were infected (data not shown). The levels of WAF 1 in the three different populations of cells are shown in Fig. 5e. Cells infected with HIV-1 NL4-3 showed reduced levels of WAF 1 compared with cells infected with HIV-1 NL4-3-nef-stop or mock-infected cells.

(ii) **Nef inhibits induction of p53 in response to UV radiation.** In normal cells p53 levels are extremely low because it is rapidly degraded (25, 35). Following UV irradiation p53 levels rise due in part to stabilization of the protein (59). Thus, we tested the effect of Nef on the induction of p53 levels following UV irradiation. Nef-electroporated MOLT-4 cells failed to increase p53 concentrations to normal levels following UV exposure (Fig. 6a, top, lane 6). The binding of Nef to p53 correlated with a loss of p53 protein induction, as Nef 1-57 prevented UV-induced accumulation of p53 (Fig. 6a, top, lane 7), while Nef 20-206 and GST had no effect (Fig. 6a, top, lanes 8 and 9). The lack of effect of Nef 20-206 on p53 levels is not due to the instability of this protein, as similar levels of Nef 20-206, Nef 1-206, and Nef 1-57 were detected in the extracts after immunoblotting (Fig. 6a, bottom). Treatment of cells with Nef 1-206 or Nef 1-57 had no effect on the levels of actin (Fig. 6a, middle, lanes 6 and 7). Analysis of p53 mRNA levels in Nef-electroporated UV-irradiated MOLT-4 cells showed equivalent amounts detected with various treatments, indicat-

FIG. 7. Nef leads to p53 destabilization. Shown is a pulse-chase analysis of p53 turnover in Nef-treated p53-expressing MOLT-4 cells. Cells were electroporated with purified full-length Nef, Nef 20-206, Nef 1-79, or Nef 1-57 or, as controls, were electroporated with GST or were mock electroporated. Following electroporation, the cells were methionine starved for 30 min, pulsed with [35 S]methionine, and chased with cold methionine for the times indicated, and protein extracts were prepared. p53 was immunoprecipitated from the extracts and separated by SDS-PAGE. The levels of actin in the extracts were also measured using antibodies against actin in immunoblotting. The levels of actin in extracts prepared from cells electroporated with Nef 1-206 or Nef 1-57 are shown.

ing that the effect of Nef on p53 levels occurred at a posttranscriptional level (Fig. 6b).

(iii) Nef enhances p53 degradation. Steady-state levels of p53 are regulated, in part, by ubiquitination and degradation (23, 29, 34). Pulse-chase experiments demonstrated that treatment of MOLT-4 cells with Nef dramatically shortened the half-life of p53 in these cells (Fig. 7). The half-life of p53 was also dramatically shortened by treatment of the cells with Nef 1-57, while Nef 20-206 and GST had no effect (Fig. 7). The regulation of p53 protein stability by Nef correlated with its ability to bind p53. Assessment of the effect of Nef derived from HIV-1 isolate C42 on p53 levels also showed that it dramatically reduced p53 levels in the MOLT-4 cells (data not shown). Again these data support the idea that degradation of p53 is a conserved function of Nef. Although the half-life of p53 in these experiments is relatively long, it is consistent with multiple reports (12, 42). Differences in p53 stability may be dependent on the cell type used.

p53 levels are reduced during HIV-1 infection. To assess whether the expression of Nef during HIV-1 infection reduces the levels of endogenous p53 and inhibits the induction of p53 by cells in response to UV irradiation, extracts of MOLT-4 cells infected with either HIV-1 expressing wild-type Nef (HIV-1 NL4-3) or virus devoid of Nef expression capabilities (HIV-1 NL4-3-nef-stop) were compared for levels of p53 before and after exposure of the cells to UV light. Levels of p53 were reduced during infection of cells with intact HIV-1 NL4-3 compared with those during infection with HIV-1 NL4-3-nef-

FIG. 8. p53 protein levels are reduced during HIV-1 infection of MOLT-4 cells with HIV-1 NL4-3. MOLT-4 cells were infected with HIV-1 NL4-3 (lanes 2 and 5) or HIV-1 NL4-3-nef-stop (lanes 3 and 6) or were mock infected (lanes 1 and 4). After a determination by immunofluorescence staining of intracellular p24 that similar numbers of cells were infected by each virus, cells either were exposed to UV light or were not treated. They were then harvested from culture, and whole-cell extracts were prepared. The extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-p53 (top) or antiactin (bottom).

stop (Fig. 8, lanes 5 and 6), while the levels of other host cell proteins examined, such as actin, were unchanged (Fig. 8). The numbers of cells infected with HIV-1 NL4-3 and HIV-1 NL4-3-nef-stop (conservatively 50% of the populations in each case) and the amounts of virus produced were similar, as identified by immunofluorescence studies and a cell-free reverse transcriptase assay, suggesting that differences in p53 levels were not due to significant differences in numbers of infected cells (data not shown). Mock-infected and HIV-1 NL4-3-nef-stop-infected cell populations also showed increased levels of p53 in response to UV irradiation (Fig. 8, lanes 1 and 3). However, in contrast, cells infected with HIV-1 NL4-3 did not show significantly increased levels of p53 after UV stimulation (Fig. 8, lane 2).

Nef expressed during HIV-1 infection can protect infected cells against UV-induced apoptosis. Previous reports by our laboratory have shown that the amounts of Nef taken up by cells during the electroporation procedure are equivalent to those expressed during HIV-1 infection *in vitro* (21). Similarly, the amounts of Nef expressed during transfection are representative of amounts of Nef expressed during HIV-1 infection *in vitro*, as determined by immunoblotting (data not shown). However, to determine whether the levels of Nef expressed during HIV-1 infection of MOLT-4 cells are sufficient to protect this cell population against UV-induced apoptosis, HIV-1 NL4-3- and HIV-1 NL4-3-nef-stop-infected MOLT-4 cells were exposed to UV light. Mock-infected cells exposed to lethal doses of UV light undergo rapid p53-dependent apoptosis, as determined by TUNEL with FITC staining (Table 3). However, HIV-1 NL4-3-infected MOLT-4 cells were resistant to UV-induced apoptosis as determined by dual staining of the cells with TUNEL-FITC and anti-HIV-1 sera (Table 3). In contrast, dual staining of MOLT-4 cells infected by HIV-1 NL4-3-nef-stop showed that the infected cells in this popula-

TABLE 3. Nef expressed during HIV-1 infection protects cells against UV-induced apoptosis^a

Infecting agent	Mean % of apoptotic HIV-1 ⁺ cells $\pm$ SEM	
	+ UV	No UV
None (mock)	69.2 $\pm$ 4.3 ^b	8.6 $\pm$ 3.4 ^b
HIV-1 NL4-3	14.4 $\pm$ 3.3	12.8 $\pm$ 2.9
HIV-1 NL4-3-nef-stop	73.8 $\pm$ 5.1	16.4 $\pm$ 3.1

^a MOLT-4 cells were infected with either HIV-1 NL4-3 or HIV-1 NL4-3-nef-stop or were mock infected. During the course of infection the cells were exposed to UV light and returned to culture. HIV-1-positive cells induced to undergo apoptosis were measured by staining by TUNEL with FITC and anti-HIV sera in conjunction with flow-cytometric analysis or immunofluorescence microscopy. The percentages of HIV-1⁺ cells undergoing apoptosis were identified by anti-HIV serum staining.

^b Percentage of the total population.

tion underwent apoptosis in response to UV light exposure (Table 3).

DISCUSSION

In this report we provide conclusive evidence showing that Nef expressed both alone and during HIV-1 infection can protect cells against UV-induced, p53-dependent apoptosis. A direct protein interaction between p53 and the N-terminal 57 amino acids of Nef mediates this effect. Binding of Nef to p53 shortens its intracellular half-life, reduces UV-induced p53 accumulation, and inhibits p53 transcriptional activation. Taken together these effects dramatically reduce intracellular p53 concentrations and activity. The ability of Nef to inhibit p53-dependent apoptotic events correlates directly with its control of intracellular p53 concentration and cell viability during HIV-1 infection of CD4⁺ T cells. The ability of Nef to inhibit apoptosis is likely to be an important feature of its capacity to enhance virus replication and by association may be related to the increased pathogenicity linked with the expression of Nef during HIV-1 infection in vivo. Nef now joins E1A, E6, and other viral proteins in modulating apoptosis through an interaction with p53 (55). The conserved function of Nef action on p53 is also supported by our studies with the *nef* genes from two primary HIV-1 isolates.

We have mapped the p53-binding domain within Nef to its N-terminal region. A fragment of Nef corresponding to amino acid residues 1 to 57 binds directly to p53 as efficiently as its full-length counterpart, while p53 binding to Nef is abolished by deletion of the first 19 amino acid residues of Nef. Hence it is possible that the precise binding region for p53 resides within the first 19 amino acid residues, although inclusion of a synthetic peptide corresponding to this region of Nef into the binding assays did not support binding to p53. Most likely the first 19 amino acid residues of Nef play an important role in stabilizing a conformation of Nef which is necessary for p53 interaction and/or the precise interactive domain requires elements near the junction of each Nef fragment (amino acid residues 1 to 19 and 20 to 206). Indeed, Nef 20-206 displays reduced binding capacity for Lck despite the fact that the binding domain for Lck resides within the proline repeat motif (amino acid residues 69 to 78) of Nef (unpublished data). While there is considerable sequence heterogeneity within the

N-terminal 57 amino acid residues of Nef, clusters of highly conserved residues, which may represent the p53 binding domain, are present. In particular, amino acid residues 18 to 32 (ERMRRRAEPAADGVGA) of Nef are highly conserved among all Nef sequences reported (52).

The activity of Nef against p53 resembles to that of MDM-2. MDM-2, a 409-amino-acid protein, plays a role in the regulation of p53 by directly binding to p53 (4). Overexpression of MDM-2 inhibits the transcriptional activation of p53 and can inhibit p53-induced cell cycle arrest and apoptosis (4), (39). Like MDM-2 (4), Nef inhibited the ability of p53 to bind and transactivate a target promoter containing the p53 consensus target DNA sequence. Nef, like MDM-2, may, by binding directly to the N-terminal region of p53 (its transactivation domain), block interaction of p53 with transcriptional coactivator proteins such as transcription factor TAF31 (57). Concurrently, reduced DNA binding activity by p53 and p53-mediated transcriptional activation may be related to its destabilization by Nef. This argument is supported by a significant decrease in the steady-state level of p53 induced by Nef. Additionally, Nef prevented an increase in p53 levels in response to stress signals such as UV radiation. Normally the p53 protein is kept at a low concentration in a cell by its relatively short half-life (25, 35). MDM-2, which promotes p53 degradation in a manner dependent on its direct interaction with p53, is thought to play a key role in maintaining low levels of p53 in normal cells (23). As Nef displays functional characteristics similar to those of MDM-2, it may promote degradation of p53 in a similar fashion.

Subcellular localization of Nef and p53 will be a determining factor in their complex formation. Detailed immunohistochemical and subcellular fractionation studies have shown that, while Nef is predominantly plasma membrane localized, significant amounts of Nef protein are also found in the cytoplasm and nucleus (27, 32, 40, 41, 43). Similarly, MDM-2 localizes to the nucleus and cytoplasm (4). Transient expression of a full-length Nef protein in 293T cells confirmed the cytoplasmic and nuclear localization of Nef (A. L. Greenway et al., unpublished data). Furthermore, colocalization studies using immunofluorescence staining for p53 and Nef expressed during transfection showed that Nef and p53 colocalize in the nucleus, whereas p53 predominates in 293T cells (A. L. Greenway et al., unpublished data). Colocalization of Nef and p53 is consistent with the interaction between Nef and p53. A Nef-EGFP fusion protein corresponding to amino acid residues 1 to 75 demonstrated a localization pattern identical to that of its full-length Nef counterpart in 293T cells, suggesting that the N terminus of Nef is sufficient to enable nuclear localization (A. L. Greenway et al., unpublished data). During HIV-1 infection nuclear localization of Nef appears to be dependent on the coincidence of the infection cycle with the peak of virus production (43). Normally, p53 is localized in the cytoplasm, the nucleus, or both subcompartments of the cell, and to execute transcriptional activation p53 must enter the cell nucleus (30, 51). The overlapping spatial arrangement of Nef and p53 may allow interaction. It is of interest that conformational changes within p53 which can be brought about by protein-protein interaction may play a major role in p53 subcellular distribution (60).

Other mechanisms for inhibition of apoptosis by Nef also exist. Nef blocks T-cell receptor (TcR)-mediated apoptosis in

CD4⁺ T cells via its interactions with *src* family kinases Lck and Fyn and MAPK (A. L. Greenway et al., unpublished data). Regulation of p53-mediated apoptosis by Nef represents a second mechanism whereby Nef can regulate the survival of the HIV-1-infected cell. In fact, modulation of *src* kinases and MAPK by Nef may represent additional mechanisms by which HIV alters p53 activity. High expression of Ras or activation of the MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest (13, 50). Similarly, in a cell line defective in both the MAPK pathway and in p53 expression, increased expression and activity of MAPK restore the normal levels of p53. This effect may be due to phosphorylation of p53 by MAPK, thereby stabilizing the protein (35, 37). The ability of Nef to regulate events which occur at the plasma membrane by targeting *src* family kinases and MAPK coupled with its ability to control p53 activity suggests that Nef may target different proteins depending upon its subcellular localization. Thus, Nef may create multiple opportunities to control normal cellular events for the benefit of virus replication.

Many oncogenic or transforming viruses encode proteins which inactivate p53 (55). For example, the simian virus 40 T antigen prevents DNA binding by p53, while the E6 protein of human papillomavirus targets p53 to the ubiquitin-dependent proteolytic pathway (55). Further still, adenovirus encodes two proteins of 19 and 55 kDa which modulate p53 activity; in particular, the 55-kDa protein interacts with p53 that is bound to its specific DNA recognition element and prevents transactivation (55). The present study highlights a further example of a viral protein which targets p53 and underscores the importance of controlling this complex transcription/tumor suppressor factor during virus infection.

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