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Mutational analysis of the human immunodeficiency virus type 1 Eli Nef function

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ABSTRACT **The studies presented here define an internally consistent experimental system that permits systematic analysis of the effect of *nef* on the rate of the human immunodeficiency virus type 1 (HIV-1) replication in a CD4$^+$ tumor T-cell line and in primary peripheral blood mononuclear cells. The parental full-length Nef protein, derived from the Eli strain of HIV-1, accelerates virus replication in both cell types. Mutations that destabilize or alter the intracellular location of the protein affect the ability of the Nef protein to accelerate virus replication. A set of mutants was made in amino acids proposed to be required for Nef function, including threonine and serine residues proposed to be targets for phosphorylation, and in sequences thought to resemble the G-1, G-3, and G-4 sites of the family of G proteins. In most cases alterations of the critical amino acids yield stable Nef proteins of parental phenotype. These results challenge the existing theories for the mechanism of Nef function. The results also identify two residues in the carboxyl half of the protein that are important for Nef function.**

The nef gene (*nef*) product is encoded by an open reading frame located at the 3′ end of the human immunodeficiency virus type 1 (HIV-1) and overlapping the U3 region of the 3′ long terminal repeat (LTR) (1). Conservation of this coding region among all HIV-1 and HIV-2 strains as well as among the simian immunodeficiency viruses suggests that *nef* plays an important role in natural infection (2, 3). The Nef protein of the simian immunodeficiency virus 239 strain was shown to be required for prolific replication in rhesus monkeys (4). The purpose of this study is to define mutations in *nef* that alter the rate of virus replication in CD4$^+$ T cells and in primary peripheral blood mononuclear cells (PBMCs).

The *nef* product, a 25- to 27-kDa protein, is modified by N-terminal myristoylation and phosphorylation and is located in the cytoplasm of infected cells (5, 6). Sequence similarity of certain regions of the Nef protein to functional domains of transducing proteins was noted (6, 7). Biochemical activities found in G proteins, including GTP and GDP binding, GTPase activity, and autophosphorylation, have been attributed to the Nef protein but have not been confirmed by subsequent studies (6, 8, 9).

The role of *nef* during virus infection has been evaluated in cultured CD4$^+$ cell lines. In some studies, isogenic viruses that differed in their ability to express *nef* displayed a slight negative effect upon virus replication (10–13). Moreover, it was suggested that the suppressive effect of *nef* was mediated through sequences located in the viral LTR (14). In studies conducted by other investigators, *nef* was shown to have either no effect or a slight positive effect upon virus replication and no influence on basal or induced LTR activity (15–17).

A HIV-1 recombinant virus (HXB-Eli 1) was characterized that is dependent upon *nef* expression for rapid replication in CD4$^+$ T cells and primary PBMCs (18). The *nef* gene used in this study is derived from the African isolate Eli (19). The effect of a selected set of *nef* mutations on virus replication in a CD4$^+$ tumor T-cell line and in primary PBMCs is reported.

MATERIALS AND METHODS

Construction of Mutant *nef* Genes. Site-specific mutagenesis was performed on single-stranded DNA using the method described by Zoller and Smith (20). The substrate for mutagenesis was a plasmid carrying the *Bam*HI (8478) to *Sac* I (9575) viral DNA fragment (Fig. 1), prepared in the CJ236 bacterial strain, as described (20). Selected amino acids of the Nef protein were altered (Fig. 2) using sequence-specific oligonucleotides.

The sequence of each altered DNA fragment was confirmed by DNA sequencing using the method described by Sanger *et al.* (21) and subcloned into a SV40 early promoter expressor plasmid. The mutant DNA was also used to replace the *nef* sequences between the *Bam*HI and *Sac* I sites of the HXB-Eli 1 provirus (Fig. 1).

Transfection of COS-1 Cells and Immunoprecipitation of Viral Proteins. Transfection of COS-1 cells with either proviral DNA or SV40 early promoter expressor plasmids was carried out as described (22). Sixty hours after transfection, the cells were labeled for 3 hr with either 100 μCi of [^{35}S]methionine per ml or 300 μCi of [^{3}H]myristic acid per ml (1 Ci = 37 GBq), lysed in RIPA buffer, and immunoprecipitated with a rabbit Nef-specific polyclonal antiserum made against the Bru Nef protein, as described (23).

Pulse–Chase Labeling of the Nef Protein. Sixty hours after transfection of COS-1 cells with 10 μg of HIV-1 proviral DNA, the cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml for 2 hr. The cells were lysed in RIPA buffer after 2, 4, and 8 hr of chase, and the Nef protein was immunoprecipitated from the extracts using a rabbit Nef-specific polyclonal antiserum.

Trypsin Digestion. A pSelect plasmid (Promega) carrying the *Bam*HI to *Sac* I viral DNA fragment was linearized at a unique *Sal* I site 3′ to the HIV insert and transcribed using T7 RNA polymerase. Translation of RNA was then performed in a rabbit reticulocyte lysate, in the presence of 0.2 μCi of [^{35}S]methionine per μl, as described (24). Translated proteins were digested with 0.1 and 1 μg of trypsin (Sigma) for 15 min at 20°C and subjected to PAGE analysis.

Chloramphenicol Acetyltransferase (CAT) Assay. Jurkat cells (10^7) were transfected, using the DEAE-dextran method (25), with either 2 μg of LTR-CAT constructs or 2 μg of LTR-CAT construct and 2 μg of a *tat* expressor plasmid.

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell; SV40, simian virus 40; CAT, chloramphenicol acetyltransferase.

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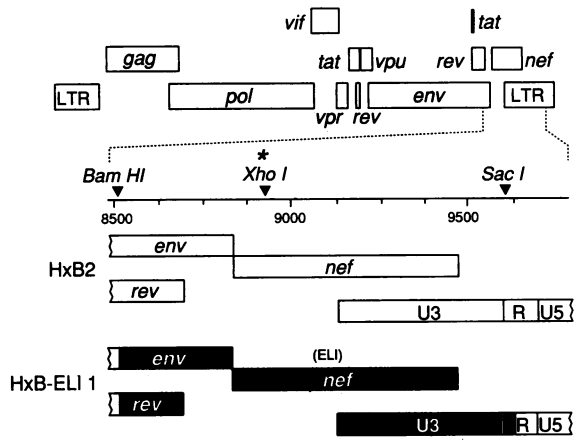

FIG. 1. Description of the recombinant HIV-1 provirus. HXB2 sequences, between sites *Bam*HI (8478) in *env* and *Sac* I (9575) in the R region of the LTR, were exchanged with the same sequences from the Eli strain. All the other viral sequences were from the HXB2 strain. The *nef*-negative virus was constructed by insertion of four nucleotides at the *Xho* I site (8900) in the *nef* reading frame. Nef-mutated proviruses were constructed by inserting the *Bam*HI to *Sac* I fragment, harboring the specific mutations, into the above described construct. Simian virus 40 (SV40)-*nef* expressors were made by inserting the viral DNA fragment from the *Bam*HI site in *env* to the end of the LTR, 3′ to a SV40 early region promoter.

Reverse Transcriptase (RT) Assay and HIV p24 Detection. Seventy-two hours after transfection of COS-1 cells with 10 μg of proviral DNA, the supernatants were harvested, cleared, filtered through 0.2-μm filters, and normalized for RT activity, as described (26). The same protocol was used for detection of RT activity in the supernatants of infected cells during the replication studies. HIV p24 core protein was detected in the supernatants of infected PBMCs using a commercially available RIA kit (NEN/DuPont).

Infection of T Lymphocytes and of Primary PBMCs. Infectious supernatants from COS-1 cells were used to infect Jurkat T cells and primary PBMCs. RT normalized supernatants (10^4 cpm) were applied to 10^6 Jurkat cells or 5×10^6 PBMCs, previously stimulated with 5 μg of phytohemagglutenin per ml (Sigma) for 3 days and kept in culture in the presence of 10 units of recombinant human interleukin 2 per ml (Boehringer Mannheim). The infected cells were monitored daily for RT activity or p24 expression and cytopathic effects up to 4 weeks after infection.

RESULTS AND DISCUSSION

Eighteen single amino acid substitutions were introduced into the *nef* open reading frame. Additionally, a frameshift mutation was introduced at the unique *Xho* I site that prematurely truncates the Nef protein at amino acid 35. A set of point mutations that change amino acids thought to be important for Nef function was made (Fig. 2). The sequence of all mutant genes was confirmed by DNA sequencing. The

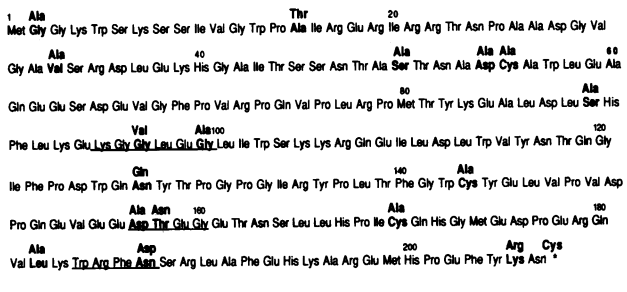

FIG. 2. Amino acid sequence of the HIV-1 Eli Nef protein. Altered amino acids and corresponding mutations are in bold type.

effect of these mutations on the promoter activity of cis-acting sequences of the LTR was examined. Moreover, all altered and wild-type Nef proteins were expressed in COS-1 cells, under the control of the SV40 promoter and in the context of the intact provirus (Fig. 1). The stability of the Nef protein in transfected COS-1 cells was also evaluated. The sensitivity to trypsin digestion of Nef proteins, produced in reticulocyte extracts from RNA made *in vitro*, was examined. The Eli *nef* of the HXB-Eli 1 viral DNA was substituted with a mutant *nef* gene. Replication of the recombinant viruses was examined in the Jurkat $CD4^+$ tumor T-cell line and in primary PBMCs.

LTR Activity of *nef* Mutants. Mutations in the region of overlap between the *nef* open reading frame and the LTR, codons 99–207, may affect functional sites of the U3 region of the LTR. To assess possible alterations conferred by *nef* mutants upon LTR activity, the *Xho* I (8900) to *Hin*dIII (9619) DNA fragment containing the entire LTR sequence was inserted 5′ to the CAT bacterial gene. The relative capacity of altered LTRs to direct CAT protein synthesis in the presence or absence of *tat*, as compared with that of the parental LTR, was examined in Jurkat cells. No differences in LTR-directed CAT enzyme activity were observed between the parental and the altered LTR sequences, indicating that the changes in the *nef* reading frame do not affect the basal or the *tat*-induced LTR activity (data not shown).

Construction, Expression, and Phenotype of the Eli *nef* Mutants. The expression of wild-type and altered Nef proteins was studied in COS-1 cells transfected with *nef* under control of the SV40 promoter or in the context of the HXB-Eli 1 virus DNA. Seventy-two hours after transfection the cells were labeled with [^{35}S]methionine. The Nef protein was immunoprecipitated from cell extracts using a rabbit polyclonal Nef-specific antiserum raised against the Bru Nef protein, made in *Escherichia coli*. The electrophoretic mobility and abundance of the protein were determined by autoradiography. Supernatants from COS-1 cells transfected with proviral DNA were used to initiate infection of Jurkat cells and primary PBMCs. Jurkat cells (10^6) and 5×10^6 primary PBMCs were infected with 10^4 cpm of virus, as measured by a standard assay for the viral DNA polymerase activity. The culture medium was replaced daily.

Expression of wild-type Nef protein results in the appearance of a 27-kDa protein (Fig. 3*A*, lane 2). The stability of the protein in a pulse–chase experiment and the partial trypsin digestion profile of the protein are shown in Fig. 4 *A* and *B*. The rapidly migrating band can be attributed to disulfide bonds that remain intact after partial reduction of the Nef protein (unpublished observations). A frameshift mutation at the *Xho* I site of *nef* abolishes expression of the protein (Fig. 3*A*, lane 1). The data of Fig. 5*A* show that the parental virus replicates faster in Jurkat cells than does the isogenic *nef*-negative virus containing a premature frameshift mutation, as evaluated by syncytium formation and the level of RT activity in the cell supernatant fluid. The peak levels of viral p24 core protein in the supernatant of infected PBMCs are delayed by 3 days (Fig. 6). The positive effect of Eli *nef* on replication of the virus in this specific genetic context is consistent with previous studies using a similar virus (18).

The most N-terminal mutation is a change of glycine at position 2 to alanine. This mutation is predicted to disrupt N-terminal myristoylation of the protein. Expression from this altered gene results in the appearance of a stable protein with a slightly higher apparent molecular weight than that of the wild-type Nef protein (Fig. 3*A*, lane 3). The altered protein is not modified by N-terminal addition of myristic acid, as evaluated by immunoprecipitation of COS-1 cells metabolically labeled with [^{3}H]myristic acid (Fig. 3*B*, lane 4). The altered Nef protein is stable and displays normal sensitivity to digestion by trypsin (Fig. 4*B*). A virus carrying this

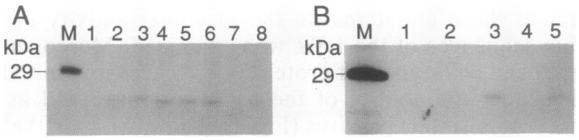

FIG. 3. Nef protein expression and myristoylation in COS-1 cells. Sixty hours after transfection of COS-1 cells with proviral DNA, the cells were labeled with 100 μCi of [^{35}S]methionine per ml for 3 hr. The cells were then immunoprecipitated with a rabbit polyclonal Nef-specific antiserum. The proteins were analyzed in an SDS/14% polyacrylamide gel (*A*). Lane 1, Nef negative; lane 2, wild-type Nef; lane 3, mutant Gly-2; lane 4, mutant Ala-15; lane 5, mutant Gly-97; lane 6, mutant Thr-158; lane 7, mutant Asp-54; lane 8, mutant Leu-182. The same protein expression was obtained when the cells were transfected with a SV40 early region promoter-*nef* expressor plasmid. Sixty hours after transfection of COS-1 cells with a SV40-*nef* expressor plasmid, the cells were labeled for 3 hr in the presence of 300 μCi of [^{3}H]myristic acid per ml and then lysed in RIPA buffer. The proteins were immunoprecipitated with rabbit polyclonal Nef-specific antiserum and analyzed in an SDS/12% polyacrylamide gel (*B*). Lane 1, mock transfection; lane 2, Nef-negative; lane 3, wild-type Nef; lane 4, myristoylation defective *nef* mutant; lane 5, mutant Ala-15. M, size markers.

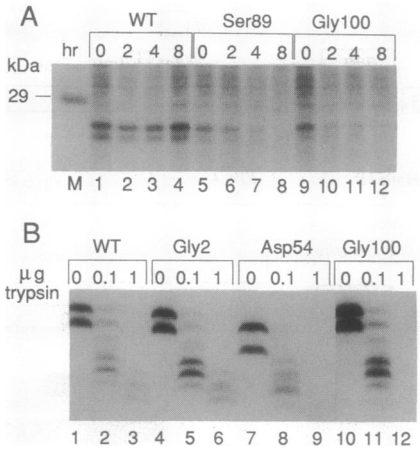

FIG. 4. Evaluation of the stability of Nef proteins and partial trypsin digestion analysis. Sixty hours after transfection of COS-1 cells with 10 μg of proviral DNA, the cells were labeled with [^{35}S]methionine for 2 hr. The medium was then replaced with medium containing unlabeled methionine. The cells were lysed after 2, 4, and 8 hr of chase and submitted to immunoprecipitation with a rabbit Nef-specific antiserum. Time 0 corresponds to the end of the pulse labeling. Proteins were analyzed in an SDS/14% polyacrylamide gel (*A*). Lanes 1–4, wild-type (WT) Nef protein; lanes 5–8, mutant Ser-89; lanes 9–12, mutant Gly-100. M, size markers. *In vitro* translated proteins were submitted to partial trypsin digestion with 0.1 and 1 μg of trypsin for 15 min at 20°C. The resulting peptides were analyzed in an SDS/20% polyacrylamide gel. Representative patterns are shown (*B*). Lanes 1–3, wild-type (WT) Nef protein; lanes 4–6, mutant Gly-2; lanes 7–9, mutant Asp-54; lanes 10–12, mutant Gly-100.

mutant *nef* gene replicates as slowly in Jurkat cells and in PBMCs as does the virus producing the prematurely truncated Nef protein (Figs. 5*A* and 6).

Threonine 15 of the Bru strain of HIV-1 is reported to be phosphorylated by protein kinase C (27). The change of alanine to threonine in the Eli strain should permit phosphorylation of the mutant Eli protein. The change from alanine to threonine does not affect the apparent size (Fig. 3*A*, lane 4) or stability of the protein. The rate of replication of viruses harboring this change is similar to that of the parental Eli *nef* virus (Table 1), indicating that phosphorylation of threonine 15 is not required for Nef function. It is also reported that extensive passage of the Bru strain of HIV-1 results in the conversion of alanine 33 to valine (28). Conversion of alanine 33 of Eli *nef* to valine has no apparent effect on the size, stability, trypsin sensitivity of the Nef protein, or its ability to accelerate the replication of the HXB-Eli 1 virus (Table 1).

Mutations that change the two serines at positions 50 and 89 to alanine should prevent possible phosphorylation of the protein at these sites (27). Substitution of alanine for serine 50 has no effect on either Nef stability, sensitivity to trypsin, or virus replication. Substitution of alanine for serine 89 results in the expression of an unstable Nef protein (Fig. 4*A*) that is abnormally sensitive to trypsin as well as in reduction of the relative rate of virus replication in Jurkat and in primary PBMCs (Table 1).

Previous studies showed that the electrophoretic mobility of *nef* alleles is markedly affected by the amino acid at position 54 (29). Fig. 3*A* shows that the electrophoretic mobility of the protein is increased by substitution of alanine for aspartic acid 54. The altered protein is stable but displays a different trypsin digestion profile from that of the parental Nef protein (Fig. 4*B*), indicating a conformational difference in their structure. Despite significant differences in the conformation of these two proteins, both Nef proteins accelerate the rate of replication of the HXB-Eli 1 virus in Jurkat cells and PBMCs (Figs. 5*B* and 6).

The three cysteine residues of the Nef protein, at positions 55, 143, and 170, were also altered. A cysteine residue was also added to the C terminus of the Nef protein. The two first cysteines are conserved among HIV-1 isolates. The third cysteine is not conserved; however, a cysteine is present at the C terminus of some Nef proteins. Neither substitution of alanine for cysteine 55 nor addition of a C-terminal cysteine affects the stability or trypsin sensitivity of the Nef protein. Viruses carrying either of these mutants replicate as rapidly as does the parental virus (Table 1), indicating that these cysteines are not required for a specific Nef function. In contrast, changes of cysteines 143 or 170 to alanine yield viruses that replicate slower than does the parental virus in Jurkat and in PBMCs. The peak viral DNA polymerase activity in the supernatant is delayed from 4 to 6 days. The peak viral p24 protein expression in the supernatant of PBMC cultures is delayed by 3 days. Substitution of alanine for cysteine 143 results in expression of an unstable Nef protein. Conversion of cysteine 170 to alanine has no effect on the stability of the protein. However, alterations in both cysteine residues result in a pattern of trypsin digestion products that is different from that produced by digestion of the parental protein (Table 1), suggesting that these cysteines play a role in determining the conformation of the protein. It is likely that cysteines 143 and 170 are required for proper folding of the Nef protein. The possibility that these two cysteines play a role in Nef function other than conformation cannot be excluded.

The two glycine residues at positions 97 and 100 are located in a conserved sequence of Nef proteins, Lys-Gly-Gly-Leu-Glu-Gly, that was proposed to resemble the GTP binding site of the G proteins (27). A change of these two glycine residues to valine and alanine, respectively, should eliminate the possibility of GTP binding in Jurkat cells. Conversion of glycine 97 to valine results in the expression of a stable Nef protein that does not reduce the replication rate of the virus. Conversion of glycine 100 to alanine yields an unstable protein (Fig. 4*A*) that displays an increased sensitivity to trypsin digestion when compared to that of the parental protein (Fig. 4*B*) and reduces the rate of replication of the virus in both cell types (Table 1). These results suggest that the proposed GTP binding consensus sequence is not required for Nef function. This result is in agreement with recent studies that fail to demonstrate GTP binding by purified Nef proteins (8, 9, 30, 31).

Several other changes were made in the C terminus of the protein. A change of the asparagine 127 to glutamine prevents

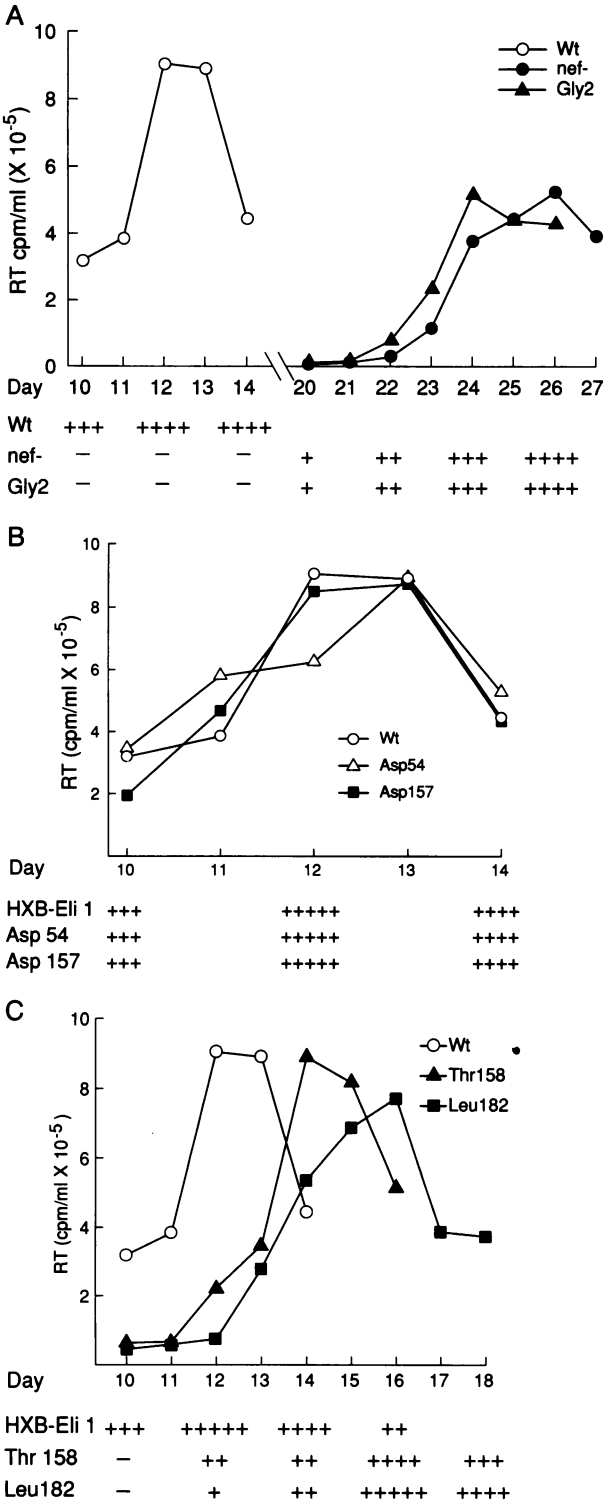

FIG. 5. Replication of recombinant HIV-1 viruses in Jurkat cells. Cells (10^6) were infected with 10^4 cpm of RT normalized supernatants from COS-1 cells transfected with 10 μg of proviral DNA. The culture medium was changed and assayed for RT activity daily. Comparative levels of syncytium formation in the cell cultures are indicated. Wt, wild type.

possible N-glycosylation of the protein at this site. Aspartic acid 157 is located in a region resembling the G-3 site, Asp-Xaa-Xaa-Gly of the G proteins. The change of aspartic acid 157 to alanine is predicted to eliminate the possible activity of this sequence. A change of asparagine 187 to aspartic acid recreates the sequence Arg-Phe-Asp-Ser found

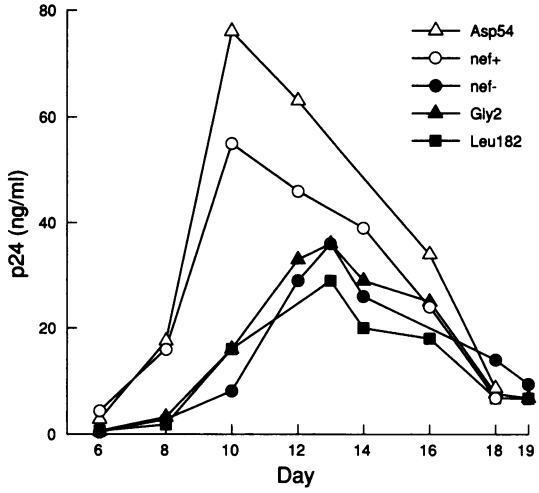

FIG. 6. Replication of recombinant HIV-1 viruses in primary PBMCs. Cells (5×10^6) were infected with 10^4 cpm of RT normalized supernatants from COS-1 cells transfected with 10 μg of proviral DNA. The culture medium was changed daily and assayed for p24 expression.

in the Nef protein of the Bru strain. This sequence was proposed to resemble the G-4 site of the G proteins (27). The lysine at position 205, conserved among Nef proteins of diverse strains, was changed to arginine. Viruses carrying mutations in the above-mentioned amino acids express Nef proteins that are stable and have the same sensitivity to trypsin digestion as that of the parental Nef protein. The rate of replication of these mutant *nef* viruses in Jurkat cells and in primary PBMCs is similar to that of the parental Eli *nef* virus (Fig. 5*B*; Table 1).

A change of threonine 158 to asparagine yields the sequence Asn-Glu-Gly-Glu. A similar sequence can be found in the Bru strain and is thought to be a putative G-4 site of the G proteins (27). The mutation that changes threonine 158 to asparagine does yield a stable protein (Fig. 3*A*, lane 6) with

Table 1. Phenotype and replication capacity of *nef* mutants

Mutation	Stability	Partial trypsin digestion	Replication	
			In Jurkat cells	In PBMCs
Wild-type	S	P	P	P
nef-negative	NM	NR	D	D
Gly-2 → Ala	S	P	D	D
Ala-15 → Thr	S	P	P	P
Val-33 → Ala	S	P	P	P
Ser-50 → Ala	S	P	P	P
Asp-54 → Ala	S	A	P	P
Cys-55 → Aa	S	P	P	P
Ser-89 → Ala	U	A	D	D
Gly-97 → Val	S	P	P	P
Gly-100 → Ala	U	A	D	D
Asn-127 → Gln	S	P	P	P
Cys-143 → Ala	U	A	D	D
Asp-157 → Ala	S	P	P	P
Thr-158 → Asn	S	P	D	D
Cys-170 → Ala	S	A	D	D
Leu-182 → Ala	S	P	D	D
Asn-187 → Asp	S	P	P	P
Lys-205 → Arg	S	P	P	P
Stop 207 → Cys	S	P	P	P

The Nef protein stability was performed in COS-1 cells transfected with either proviral DNA or SV40-*nef* expressor plasmids giving similar results. S, stable; U, unstable; P, parental; A, altered; D, delayed; NM, not made; NR, not relevant.

parental sensitivity to trypsin digestion. This change appears to decrease Nef function, as judged by the delayed replication of the virus carrying this mutation in Jurkat cells (Fig. 5C) and in PBMCs (Fig. 6). These observations indicate that it is unlikely that the regions of Nef proteins that are similar in sequence to regions of G proteins are required for acceleration of growth of the HXB-Eli 1 virus. It is possible that the mutation in amino acid 158 affects a function of the protein not related to GTP or GDP binding. This observation is consistent with reports that Nef proteins purified from other alleles fail to bind GTP or GDP and do not display a GTPase activity (8, 9, 30, 31).

A leucine residue at position 182 is present in all HIV-1 isolates sequenced to date. Changes in this amino acid result in the expression of a stable protein (Fig. 3A, lane 8) that displays parental sensitivity to trypsin digestion. Nonetheless, viruses that contain *nef* substituted at leucine 182 replicate more slowly than does the parental virus. The peak of viral DNA polymerase and the number of syncytia are delayed from 4 to 6 days in Jurkat cell cultures (Fig. 5C). The peak of p24 Gag protein expression in cultures of PBMCs is delayed by 3 days (Fig. 6). The observation that a conserved amino acid substitution, alanine for leucine, decreases the activity of the protein highlights the critical nature of leucine 182 for Nef function.

The lack of a consistent means for measuring the effect of Nef on virus growth rate has prevented a systematic analysis of the genetics and the biochemistry of this protein. The results presented here establish an internally consistent assay for the effect of Nef on HIV-1 replication in the $CD4^+$ tumor T-cell line Jurkat, and in primary PBMCs. The results also identify residues of Eli *nef* important for rapid replication and challenge existing theories for the biochemical activities required for Nef protein function.

A previous study demonstrated that the Eli *nef* allele accelerates replication in Jurkat cells and in primary PBMCs when recombined with the HXB2 provirus, derived from the IIIB strain of HIV-1, to yield HXB-Eli 1 (18). The data presented here show that acceleration of growth by Eli *nef* in this virus strain is either abrogated or attenuated by changes in the sequence of the *nef* gene that create a premature stop codon or that demonstrably decrease the stability of the protein in infected cells. All three changes that destabilize the altered Nef protein reduce the rate of growth of the HXB-Eli 1 recombinant virus in Jurkat cells and primary PBMCs. Additionally, a mutation that alters the glycine at the N terminus of the protein and that eliminates cotranslational addition of the 14-carbon fatty acid chain, myristic acid, to the N terminus of the protein also eliminates the positive effect of the Eli *nef* allele on virus growth. Previous studies demonstrated that such a change prevents membrane association of the Nef protein (32). This study confirms the importance of N-myristoylation for Nef function. The most severe effect on HIV-1 replication in Jurkat cells occurs when the Nef protein is either prematurely truncated or is not myristoylated. Other changes, including mutations that destabilize the Nef protein, reproducibly slow virus replication but do not have the same marked effect as do the first two types of mutations. It is possible that these changes reduce but do not eliminate Nef function. A positive role for Nef in HIV-1 replication is consistent with the requirement of Nef for prolific growth of the 239 strain of simian immunodeficiency virus in rhesus monkeys (4).

Mutations in amino acids proposed to be targets for phosphorylation and in sequences thought to resemble functional sites of the G proteins do not affect the ability of the Eli Nef protein to accelerate virus replication in Jurkat cells and primary PBMCs. These observations suggest that it is unlikely that the regions of Nef similar in sequence to functional sites of the G proteins are required for function. The experimental system described here should also assist a correlation of biochemical activity with biological function of Nef proteins by investigation of the properties of purified parental and mutant Nef proteins.

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