The human immunodeficiency virus type 1 (HIV-1) early gene product Nef is a multifunctional protein that alters numerous pathways of T-cell function, including endocytosis, signal transduction, vesicular trafficking, and immune modulation, and is a major determinant of pathogenesis. Individual Nef functions include PK-2 activation, CD4 downregulation, major histocompatibility complex (MHC) class I downregulation, and enhancement of viral particle infectivity. How Nef accomplishes its multiple tasks presents a difficult problem of mechanistic analysis because of the complications associated with multiple, overlapping functional domains in the context of significant sequence variability. To address these issues we determined the conservation of each Nef residue based on 1,643 subtype B Nef sequences. Mutational analysis based on conservative substitutions and Nef sequence data allowed us to search for amino acids on the surface of Nef that are specifically required for PK-2 activation. We found residues 85, 89, and 191 to be highly significant determinants for Nef's PK-2 activation function but functionally unlinked to CD4 and MHC class I downregulation or enhancement of infectivity. These residues are not conserved across HIV-1 subtypes but are confined to separate sets of surface elements within a subtype. Thus, L85/H89/F191 and F85/F89/R191 are dominant in subtype B and subtype E or C, respectively. Our results provide support for developing subtype-specific interventions in HIV-1 disease. In an effort to derive mutants specifically defective for Nef activities that are thought to contribute to in vivo pathogenesis and disease progression, multiple laboratories have attempted to define which surface amino acids are required for each independent Nef activity by alanine-scanning mutagenesis studies (1, 26, 53). Other mutational studies have targeted novel features of Nef sequences like di-amino acid repeats and amino acids presumed to be highly conserved (2, 37, 42, 43, 47). In this latter case, Nef mutants with residues that have drastically altered chemical properties have been employed. Mechanistic conclusions based on these inactivating mutations have been supported by claims that a particular mutated amino acid residue is highly conserved, but conservation was based on compilations of relatively small numbers of different Nef sequences (16, 42, 43). A common result of these mutational approaches has been the generation of Nef mutant proteins that have multiple functional defects. One of the most intensely investigated Nef functions is activation of PK-2 by Nef (6). Several investigators have concluded that two surface loci are mechanistically crucial for PK-2 activation: the SH3 binding domain (P72 to R77) and the proposed dimerization domain bracketed by R105/R106 and D123 (see Fig. S1, top, in the supplemental material). Mutations of the SH3 binding domain (13, 22, 53) or the dimerization domain have consistently resulted in Nefs defective for multiple activities (5, 12, 17, 26, 35, 51–53). Specifically, mutations of the SH3 domain and dimerization domain result in Nefs defective for MHC class I downregulation, PK-2 activation, and enhancement of virus particle infectivity, with the dimerization domain also defective in CD4 downregulation (5, 35). To begin to resolve the mechanistic confusion resulting from these complex results, we have conducted a detailed mutational analysis of the crucial flanking amino acids of the dimerization domain.
The complexity of Nef structure/function led us to conclude that an alternative approach to mutational analysis was required for investigating Nef's multiple functions and ultimately the specific roles in pathogenesis and disease progression. To this end, in a previous report we characterized seven primary isolate Nefs for each of the four genetically separable functions listed above (17). In this way we hoped to utilize the structural integrity of functioning Nefs to discover mechanistically interpretable structure-function relationships. Nefs defective in only one of the four functions were found, and the polymorphisms in the primary isolate Nef sequences were employed for structure-function analysis. One of the polymorphisms we analyzed was a relatively subtle F-to-I mutation at amino acid position 191 in HIV-1 Nef,133 (17). This mutation greatly reduced Nef's ability to activate PAK-2 but left CD4 and MHC class I downregulation and infectivity enhancement intact. We considered this PAK-2 activation defect to be mechanistically distinct from the dimerization region, because it is located on the opposite side of Nef (see Fig. S1 in the supplemental material). Therefore, detailed mutational analysis was initiated to uncover amino acids interacting with F191 in addition to mutational analysis of the dimerization region.

To conduct these two mutational analyses, we constructed a comprehensive collection of 1,643 subtype B Nef sequences. The large number of sequences permitted the conservation of each amino acid residue of subtype B Nef to be evaluated. This knowledge enabled a mutational investigation of Nef function based on the natural selection to which the protein is subjected and led us to the new finding that Nef is subject to a dynamic evolution whereby multiple compensatory mutations maintain the PAK-2 activation function despite mutation of surface residues critical for this function. Nefs from different subtypes exhibit different sets of surface residues that nonetheless maintain Nef's multiple functions. Remarkably, these Nefs have all appeared in viruses derived from a single zoonosis thought to have occurred in the 1930s (31, 32).

MATERIALS AND METHODS

Construction of a subtype B HIV-1 Nef amino acid sequence database. The HIV-1 subtype B Nef sequence database was constructed from a BLAST search of the NCBI translated database with SF2Nef protein sequence in December 2002 (3). Nefs with non-ATG initiation codons, early termination codons, duplications of more than 60 bases, and large deletions were discarded. A few Nefs with multiple nucleotide ambiguities were discarded as having low-quality sequence data. Nefs with one or two ambiguities were assigned the most common amino acid at that position consistent with the other two codon positions so as to not lose the information in the other 99% of the sequence. Only Nefs that were 200 residues or longer were included. Out of a total of 2,405 available Nef sequences in the NCBI database, 291 were judged to be grossly defective subtype B Nefs and 554 were judged to be non-subtype B Nefs and subtype B recombinants. The large number of grossly defective Nefs in the NCBI database (15%) has been previously reported (15, 19, 25, 38, 49). We based the determination that a given Nef was subtype B on the submitter's designation or the geographical location of the isolate. This left 1,660 Nef sequences. Seventeen Nefs were found to yield stronger homology with non-B Nefs when individually blasted against the translated NCBI database and were removed. The collection of sequences was halted in December 2002 because most of the 17 Nefs removed from the database were of recent origin. This suggested that our major criterion of geographical location for subtype B identification was breaking down with the spread of several of the subtypes across the globe (31). We view this database as potentially useful for comparison with subsequent collections of Nef sequences in order to investigate ongoing sequence evolution. We also constructed an exhaustive subtype E Nef database from the limited number of subtype E Nef submissions available in December 2002.

There were 22 subtype B Nefs from 195 to 199 amino acids long, of which 19 were either 197 or 198 amino acids long. The largest impact on the database by not including the short Nefs was the removal of 10 deletions of the diacidic (E154/E155) motif. We also discarded the “V” sequences of Delassus et al., since these sequences were from virus maintained in culture to determine changes that result from in vitro passage (15).

We have not created entries that are consensus sequences from closely related sequences for the following reasons: (i) we consider variation (including separate clones) within Nef sequences from a single patient at a single time point to be of interest, (ii) we consider variation in Nef sequences from a single patient at different time points to be potentially significant, and (iii) variation in closely related sequences from direct transmission of HIV-1, such as mother-infant transmission or blood donor cohorts, is also interesting. For these reasons, we included all sequences instead of making arbitrary decisions regarding sequence relatedness. Further, HIV-1 amino acid variation does not necessarily represent amino acid substitutions but includes random mutation. By including all sequences and expanding the size of the database, we limit the significance of random mutation. Our choice to expand the size of the sequence collection by including sequences with various degrees of relatedness introduces bias that can give a false impression of the natural frequency of rare mutations. This bias is readily assessed by inspecting the NCBI entries for a given set of sequences which contain a rare amino acid for a given position. In this way, we note which minimally represented amino acids are scattered throughout the collection of sequences and which arise from one or a few patients. For example, the 21 R191s come from three patients with nine (including accession number AAA87489), six, and six sequences each. This strongly indicates that the 21 R191s do not represent random mutations. The collection of 1,643 subtype B and 70 subtype E Nef amino acid sequences in FASTA format is available upon request. Additional details of Nef sequence variability and construction of the database are presented in the supplemental material.

Cell lines and culture conditions. Human CEM T cells were transduced to express only the neomycin phosphotransferase gene (LXSN) or else nef and the nea resonance genes (L nef SN) as previously described (18). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone), 50 IU of penicillin per ml, 50 µg streptomycin per ml, 50 µg L-glutamine, 2 mM sodium pyruvate. 293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 50 IU of penicillin per ml, 50 µg of streptomycin, and 2 mM L-glutamine. Cell lines were maintained at 37°C in a humidified incubator with 5% CO2 (CEM) or 10% CO2 (293T).

Plasmid expression constructs and transfections. Desired alterations in nef sequences were made by use of the QuikChange site-directed mutagenesis kit from Stratagene and standard molecular biology techniques. DNA encoding Nefs based on accession numbers AAA87489 and AAD48623 was synthesized by Blue Heron Biotechnology. CM235 Nef was cloned into pLXSN as previously described (18). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone), 50 IU of penicillin per ml, 50 µg of streptomycin per ml, 50 µg L-glutamine and 2 mM sodium pyruvate. 293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 50 IU of penicillin per ml, 50 µg of streptomycin, and 2 mM L-glutamine. Cell lines were maintained at 37°C in a humidified incubator with 5% CO2 (CEM) or 10% CO2 (293T).

Western blot analysis. Nef expression in cell lysates was determined with sheep anti-SF2Nef serum (17) or sheep anti-CM235Nef serum (1:2,000 dilution), followed by horseradish peroxidase (HRP)-conjugated anti-sheep immunoglobulin G (IgG; 1:10,000; Chemicon International). HRP conjugates were visualized by using enhanced chemiluminescence (Amersham). The anti-CM235Nef hyperimmune serum was induced by multiple injections of a maltose binding protein/CM235Nef fusion protein (New England Biolabs). Anti-SF2Nef serum was specific for subtype B Nefs, while anti-CM235Nef serum was specific for subtype E Nefs.

Flow cytometry analysis. For analysis of cell surface CD4 and MHC class I levels, transduced CEM cells (500,000) were first incubated with mouse monoclonal anti-haplo-type A1, A11, and A26 MHC class I antibody (One Lambda) for 20 min on ice, and then the cells were washed twice in 2 ml of ice-cold phosphate-buffered saline containing 5% calf serum and 0.1% NaCl. Cells were then incubated with fluorescein isothiocyanate-labeled rabbit anti-mouse IgG for 20 min on ice. Cells were washed as indicated above, incubated for 20 min on ice with 2 µg of mouse IgG, and washed again. Cells were then incubated with phycoerythrin (PE)-conjugated IgG1 monoclonal antibody to human CD4 (Exalpa) and incubated for 20 min on ice. Stained cells were washed as indicated above and analyzed on a Becton Dickinson FACSCalibur instrument equipped with CellQuestPro software. All fluorescence data were collected in log
mode. CEM cells transduced with LXSN served as the positive control. For negative controls mouse isotype antibody replaced anti-MHC class I, and PE-conjugated mouse IgG1 (Exalpha) replaced PE-conjugated anti-CD4.

In vitro kinase assay.
The PAK-2 activation assay was performed as previously described (17, 36). Immunoprecipitations were performed using the sheep anti-SF2Nef serum except when pcDNAHA-PAK-2 was cotransfected. For those experiments, mouse monoclonal anti-HA antibody (Covance) was used. Nef/PAK-2 complexes were immunoadsorbed and, after stringent washing which included a 1M MgCl2 wash, the PAK-2/Nef protein complexes were assayed for autophosphorylation activity. Phosphorylated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and dried gels were then exposed to a phosphorimager screen (Packard).

FIG. 1. Compilation of subtype B Nef sequences. The amino acid positions 1 through 206 of the Nef protein are shown. For each position, the number of different sequences containing the indicated amino acid residue is shown followed by its letter designation. Gaps are represented as a dash. Adding up all the different residues and gaps observed for each position totals 1,643, the number of sequences analyzed.
TABLE 1. Hypermutable positions in which the most common residue represents less than half of the observed total\(^a\)

| AA position | AA composition |
|-------------|----------------|
| 8............. | 660R, 560S, 143C, 89-, 38H, 37N, 30P, 23G, 21L, 11M, | 81, 4K, 4W, 3V, 1Q, 1Y |
| 10............ | 620V, 252M, 186-, 182G, 124H, 107L, 49R, 41K, 33A, | 13T, 11C, 11E, 10F, 25, 1P, 1Q |
| 11............ | 510V, 286G, 186I, 179P, 155-, 95S, 71F, 56A, 44D, 16T, | 14N, 8R, 7E, 7L, 3M, 3C, 2K, 1W |
| 15............ | 798A, 67IT, 51K, 30N, 26R, 25S, 221V, 9V, 6G, 2L, 1D, | 1E, 1H |
| 85............ | 797L, 582V, 112I, 90F, 45R, 14M, 11W, 4H, 3S, 2A, 1C, | 133, 722I, 759T, 298V, 35P, 3AIF, 1L, 1S |
| 182.......... | 648V, 321E, 315M, 213Q, 52A, 34I, 30K, 12T, 9L, 7R, | 1G, 1P |
| 194.......... | 806M, 553V, 110K, 68I, 43R, 31L, 23T, 7F, 1A, 1E |

\(^a\) Deletions are represented as a dash. All positions add up to 1,643 AA, amino acid.

Provirus plasmid construction. To generate SF2 proviruses with mutant Nefs, the XhoI-BspEI fragment from the nef gene of the SF2 proviral molecular clone, pPB18, was replaced with the same fragment from the mutant Nef constructs by triple ligation. For subtype E/SF2 chimeric proviruses, the subtype E nef was adapted to have Bpi and BspEI restriction sites by PCR before cloning. This results in the replacement of SF2Nef amino acids 25 to 200 with the corresponding amino acids from subtype E Nefs. In all cases the corresponding chimera or mutant was confirmed by DNA restriction analysis and DNA sequencing.

Production of HIV-1$_{SF2}$ and chimeric viruses. Infectious virions of HIV-1$_{SF2}$, HIV-1$_{SF2}$ with Nef mutants, and HIV-1$_{SF2}$ subtype E Nef chimeras were produced in 293T cells (10$^6$) following transfection with p9B18 or its chimeric derivatives (2$^2$), using Lipofectamine (Life Technologies). Virus was harvested 48 h posttransfection, filtered through a 0.45-$\mu$m filter, and stored frozen in aliquots at -70°C. Lysates from the transfected 293T cells were used for analysis of Nef expression by Western blot analysis.

Infectivity assay. HeLa-MAGI cells (60,000) were plated in 12-well plates and infected 24 h later in triplicate with 5 ng of p24Gag, and stored frozen in aliquots at -70°C. At 24 h after inoculation, 1.6 ml of culture medium was added, and the cells were incubated for an additional 36 h. The cells were then fixed and stained as described previously (29). Blue cells were counted as indicators of infected cells.

RESULTS

Nef amino acid sequences display extensive polymorphism (49). There are two levels of diversity for HIV-1 Nefs. Considerable differences exist between the nine different subtypes of group M HIV-1 Nefs (31, 41). There is also much diversity within a subtype (10, 25, 30, 38, 49). The vast majority of studies of Nef function have investigated subtype B HIV-1 Nefs, but even within subtype B sequences the extent of variation is difficult to reconcile with the numerous reported activities of Nef (21, 45).

As a result of this sequence variability, crucial Nef functions have been reported to depend on amino acids that are not highly conserved (16, 42, 43, 47). To avoid mechanistic uncertainties in our mutational analyses of Nef function, we created an extensive Nef sequence collection ($n = 1,643$ sequences) and counted the amino acids present at each position of the protein (Fig. 1; see also Fig. S2 in the supplemental material).

Nef is a highly variable protein. Analysis of each of the 206 amino acids in Nef indicated that 63 positions are highly conserved at 99% (Fig. 1). Conversely, we identified eight hypermutable positions where the most common amino acid represents less than half of the total (Table 1). These eight positions indicate the intense mutational pressure on subtype B Nef during the 50 to 70 years of the protein’s existence (32, 56).

The remaining two-thirds of Nef residues are variable to different degrees, a fact that makes mechanistic interpretation of mutational analysis data exceedingly complex. For example, previously described motifs categorized as functionally important, such as E154/E155 (diacidic), R105/R106 (diarginine), and E62/E63/E64/E65 (polyacidic), have been described as highly conserved (16, 21, 24, 42, 43). However, as seen in Fig. 1, they exhibit significant variability in at least one of the residues.

More detailed analysis of the information present in the Nef database indicated that there are eight sites where the two most common residues together account for 99% or more of the total, with the second most common residue accounting for at least 10% of the total (Table 2). Consistent with the findings of Dayhoff et al., most of these pairs consist of acidic, basic, hydrophobic, or aromatic amino acids, and the conservation of each pair is likely based on structural similarity (14). In each of the eight cases the other 18 amino acids must significantly disrupt Nef function, since they are strongly selected against (%). It is our view that a similar consideration should be applied to residues in Nef that are highly conserved versus the other 19 amino acids. In this case, any mutation made for the purpose of structure-function analysis represents an amino acid that has been selected against in nature; therefore, conservative substitutions and mutations suggested by the database are used in this report.

The highly conserved R106 residue of Nef represents a structural element involved in multiple overlapping structural-functional domains. In earlier reports R106 had been mutated to L or A, and a loss of PAK-2 activation by Nef was observed (37, 46, 53). As a result, these mutations have been used by different authors to argue for PAK-2 involvement in the anti-apoptotic effect of Nef, impairment of thymopoiesis, and the in vivo pathogenesis of simian immunodeficiency virus (20, 48, 50, 55). However, mutating the highly conserved R106 (99%) to potentially disruptive mutations (L or A) may yield anomalous results (14). For this reason we compared the L and A mutations to two additional mutations, R106Q and R106K. Consistent with previous reports (17, 53), we found R106L to be entirely defective for CD4 downregulation, MHC class I downregulation, and PAK-2 activation (Fig. 2A, left, and B). R106A exhibited partial function for CD4 and MHC class I downregulation but was defective for PAK-2 activation and enhancement of infectivity. R106Q was partially functional for PAK-2

TABLE 2. Positions in which the most two common residues account for 99% of the observed total\(^a\)

| AA position | AA pair |
|-------------|---------|
| 16........... | V/I     |
| 33............ | V/A     |
| 108.......... | D/E     |
| 114.......... | V/I     |
| 116.......... | H/N     |
| 120.......... | Y/F     |
| 170.......... | L/Q     |
| 184.......... | K/R     |

\(^a\) AA, amino acid.
activation, CD4 downregulation, and enhancement of infectivity but defective for MHC class I downregulation. The least disruptive mutation at this position was R106K. This mutation exhibited weak MHC class I downregulation but was partially functional for CD4 downregulation and PAK-2 activation. The clearly distinguishing feature of R106K relative to R106A was its fully intact ability to enhance infectivity (Fig. 2C). Therefore, R106A cannot be used to distinguish between the Nef functions for PAK-2 activation and enhancement of infectivity. As a result, the linkage of Nef’s activation of PAK-2 to multiple Nef functions is premature, since the mechanisms responsible for the enhancement of infectivity function may be involved (20, 48, 50, 55). Nor can residue R106 be described as exclusively important for PAK-2 activation and enhancement of infectivity, since the R106Q mutation most severely impacts MHC class I downregulation (Fig. 1). We have previously demonstrated that Nefs with K at 105 are fully functional (17). R105 has been previously mutated to A, yielding a partial defect in CD4 downregulation (26). D123 has been mutated to A, G, V, and L, and defects were observed in all four of the Nef functions we assayed (5, 12, 35, 51, 52). To reevaluate the significance of these findings, we made two new mutations, R105Q and D123E. We based our choice of substitutions on Q being the third most frequently found residue for position 105 (6%) and E being structurally related to the highly conserved D123 (Fig. 1). We also characterized R105A (26).

**Mutational analysis of the D123 and R105 residues of SF2 Nef.** Amino acid residues D123 and R105 form salt bridges in the proposed dimeric structure of Nef (4). Mutants of D123 exhibit multifunctional defects attributed to disruption of Nef dimers (35). From the data shown in Fig. 1, we noted that D123 is highly conserved (99%) but that R105 is not. In fact, 71% of Nefs in the database are lysine at 105 (Fig. 1). We have previously demonstrated that Nefs with K at 105 are fully functional (17). R105 has been previously mutated to A, yielding a partial defect in CD4 downregulation (26). D123 has been mutated to A, G, V, and L, and defects were observed in all four of the Nef functions we assayed (5, 12, 35, 51, 52). To reevaluate the significance of these findings, we made two new mutations, R105Q and D123E. We based our choice of substitutions on Q being the third most frequently found residue for position 105 (6%) and E being structurally related to the highly conserved D123 (Fig. 1). We also characterized R105A (26).

SF2NefD123E was found to have greatly reduced activities of CD4 downregulation, MHC class I downregulation, and
enhancement of infectivity (Fig. 2B and C). Remarkably, we found SF2NefD123E to strongly activate PAK-2 (Fig. 2A, right). Different results were obtained for R105Q and R105A, which were both partially active for all four Nef functions, except that R105A was fully functional for enhancement of infectivity (Fig. 2). Thus, the use of a Nef database to choose an amino acid substitution that is actually present in 101 Nef sequences (Q105) and the substitution of a highly conserved amino acid for the structurally related amino acid (D123E) uncovered strikingly different results for two amino acids (K105 and D123) proposed to be required for Nef oligomerization (35). Since we were unable to mechanistically link K/R/Q105 or D123 to PAK-2 activation, we then investigated F191.

Structural requirements for activation of PAK-2 by Nef. We previously found F191I to be a mutation that specifically affected Nef-induced PAK-2 activation, having no effect on other activities (CD4 and MHC I downmodulation and infectivity enhancement) (17). In subtype B Nefs, the most prominent non-F residues at this position are 21 Rs, 41 Ls, and 21 Ys (Fig. 1). While F to I is a relatively mild change (compared to non-F residues at this position are 21 Rs, 41 Ls, and 21 Ys at position 191 would not be able to activate PAK-2. Conversely, compensating mutations may exist that maintain this function.

Structural requirements for activation by Nef are different between subtype B and subtype E Nefs. In addition to the 21 subtype B Nefs with R191, we noted that subtype E Nefs have almost exclusively R at this position (see Fig. S3 in the supplemental material). By analogy to our results with subtype B Nefs, this would imply that virtually all subtype E Nefs are defective for PAK-2 activation. Evaluation of PAK-2 activation, CD4 downregulation, and enhancement of infectivity by five different subtype E Nefs (CM235, 93JPNH1, 93JPNH2.5, 93TH253, and 90CF402) showed them all to be functional (Fig. 4A, B, and C). Varying capacities to downregulate MHC I were observed (Fig. 4B), consistent with previous results (11, 17). The fact that all five subtype E Nefs are functional for PAK-2 activation despite the presence of an R at position 191 suggests the presence of compensatory mutations in subtype E Nefs and indicates that subtype E and B Nefs have different structural requirements for this activity.

Characterization of the structural requirements for PAK-2 activation by R191-containing Nefs. The minimal change of F191Y inactivates SF2Nef-induced PAK-2 activation, but the drastic ortholog of R for F in subtype E Nefs leaves PAK-2 activation intact. These results indicate that compensating mutations exist within subtype E Nefs that allow PAK-2 activation to occur within the structural context that maintains the other Nef functions. To assess the PAK-2 activation function of the subtype B Nefs with R191, one of these genes was chosen for chemical synthesis (isolate RP-1-9; accession number AAA87489) (see Materials and Methods). This Nef was found to be active for PAK-2 activation (Fig. 5A, left). To find compensating mutations for R191, we relied on differences between the subtype B and subtype E sequences as well as the 21 subtype B Nefs that have R191 (Fig. 1; see also Fig. S3 in the supplemental material). We found 42 differences between the consensus sequences derived from our subtype B and subtype E databases which describe “subtype E-like” residues. We then inspected the 21 subtype B Nefs with R191 for the presence of these “subtype E-like” residues. While 97% of our subtype B Nefs are H89, the 18 of 21 Nefs with R191 were F89. All 70 subtype E Nefs sequences were also F89. Based on this information we attempted to create a “subtype E-like” surface in subtype B SF2Nef. However, when tested, the double mutant SF2NefH89F/F191R was inactive for PAK-2 activation (Fig. 5A, right). Various other “subtype E-like” residues in the 21 subtype B Nefs with R191 were then tested in preliminary assays. Ultimately, we focused on positions 85 and 188 in addition to 89 and 191. Substituting residues prevalent in subtype E Nefs into the subtype B SF2Nef yielded SF2NefL85F, SF2NefH89F, SF2NefK188A, and SF2NefK188A. All four failed to activate PAK-2 (Fig. 5A) while maintaining the ability to downregulate CD4 and MHC class I (Fig. 5B). The triple mutation H89F/K188A/F191R was also defective in its ability to activate PAK-2, but clear restoration of PAK-2 activation was found with the triple mutation L85F/H89F/F191R and with the quadruple mutation L85F/H89F/K188A/F191R. Upon examination of the three-dimensional structure of Nef (24), we noticed that residues 85, 89, 188, and 191 lie in close proximity on the protein surface. Thus, L85F/H89F/K188A/F191 (SF2Nef) and F85A/F89A/K188A/F191 represent alternative surface elements that affect the same Nef function, PAK-2 activation. The introduction of the subtype E-like F85A/F89A/K188A/F191 motif on the subtype B SF2Nef resulted in a protein capable of downregulating CD4 or MHC class I and also enhancing infectivity over its Nef-deleted counterpart (Fig. 5). The same multifunctional pheno-
type was observed for SF2NefF191R, except that this single mutant was defective for PAK-2 activation. This demonstrates the specificity for the PAK-2 inactivation of the L85F, H89F, K188A, and F191R mutations in SF2Nef.

A similar analysis was performed for the Nef F191Y mutant based on the 21 subtype B Nefs that are Y191. Six Nef isolates from a single patient with Y191 were found to harbor R85 and F89. The gene for one of these clones (accession number AAD48623) was chemically synthesized and found to be functional for PAK-2 activation (not shown). Introducing these mutations into SF2NefF191Y yielded an R85F89K188Y191 motif that restored PAK-2 activation to the defective single mutant (Fig. 6).

Additional complexity is observed upon inspection of subtype C Nef sequences. Most subtype C Nefs are R191, but about 20% are H191 (40). Most commonly, subtype C Nefs that contain H191 have F85/F89/H188. Incorporating these amino acids into the inactive SF2NefF191H yielded an F85F89A188R191 motif and restored PAK-2 activation (Fig. 6). Thus, we have evidence suggesting a fourth motif involved in the activation of PAK-2.

Structural requirements for PAK-2 activation in subtype B Nefs. The subtype B PAK-2-activating structural motif requires F at 191, since Y191, L191, I191, H191, and R191 are all inactive (Fig. 3 and 6). In Fig. 1, residue 89 is 97% H, and none of the 30 sequences with F89 are F191. Similarly, 99% of the 1,539 Nefs with F at position 191 have an H89 residue. In contrast to H89 and F191, position 85 is one of the eight hypermutable positions of Nef, with its most prevalent amino acid, L, representing less than half of the total (Table 1). We previously determined that a Nef with V at position 85 is fully functional (17). Also, the brain-derived HIV-1 isolate YU-10 has a Nef with M85 (34). This Nef, as well as SF2Nef L85M, activates PAK-2 and down-regulates CD4 and MHC class I (not shown). Thus, the proper designation for the structural requirement at position 85 is /H9278, indicating a hydrophobic residue. However, the aromatic F at this site greatly reduces PAK-2 activation (Fig. 5A). The structural requirements at position 188 remain to be fully defined.

Though SF2Nef has K at position 188 and is functional, most subtype B Nefs have an R at position 188. We have previously reported several Nefs harboring an R188 residue to be functional for PAK-2 activation (17) and more recently also found SF2Nef K188R to activate PAK-2 (not shown). The presence of 152 Hs and 119 Ss at position 188 suggests that these residues will also be functional (Fig. 1). One striking difference between the SF2Nef and SF2Nef with a subtype E-like motif (F85F89A188R191) is that SF2Nef K188A has reduced PAK-2 activation function relative to SF2Nef, while SF2NefL85F/
H89F/F191R and SF2NefL85F/H89F/K188A/F191R exhibit little difference in activity (Fig. 5).

**DISCUSSION**

Nef has been aptly described as the HIV-1 protein we understand the best and the least (23). In our view there are two major reasons for this situation of much data and little understanding. First, mutational analysis has not been database driven, which dissociates mechanistic interpretations from the underlying reality of the selection pressure to which the protein is subjected. The second issue is that, with the exception of the Nef/DOCK2/ELMO1 complex identified by Janardhan et al. using a C-terminal-tagged Nef (27), strong binding affinity of Nef to target proteins has not been demonstrated. Specific examples include the diacidic motif (E154E155) and -COP, the polyacidic motif (E62E63E64E65) and PACS-1, and the dimerization region and thioesterase (9, 28, 35, 42, 43). On the basis of limited mutational analysis, these interactions between Nef and host cell proteins have been ascribed functional significance (9, 28, 35, 42, 43). An extensive mutational analysis has been performed on the linkage of the CD4 downregulation function to thioesterase binding to Nef by Cohen et al. (12).

![FIG. 5](image_url)

![FIG. 6](image_url)
Those authors found that the binding of Nef to thioesterase was dependent on the weakly conserved D at position 108 (Fig. 1; see also Fig. S3 in the supplemental material). Substituting E for D at this position yields a Nef that fails to bind thioesterase but is fully functional for CD4 downregulation.

Similar considerations of weak affinity binding and weak conservation apply to the self-association of Nef. Arold et al. (4) described Nef dimers and trimers in vitro and suggested that these Nef homomers are linked through homologous contacts between amino acids R105, D108, I109, L112, Y115, H116, F121, P122, and D123. These amino acids are described as conserved, but this is not the case for R105, D108, or H116 (Fig. 1). Furthermore, these in vitro binding studies found no dimers below 100 μM Nef (4). Liu et al. (35) reported dimers of CD8Nef fusion proteins isolated from transfected 293T cells that were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis after denaturation under reducing conditions. We (unpublished data) and others (8, 46) have not observed these dimers. Unable to directly observe the reported dimers, we have turned to mutational analysis to find a functional linkage for R105 and D123, which are proposed to form a critical salt bridge stabilizing Nef oligomerization (4, 35). Our data (Fig. 2) do not support this linkage. Of potential interest is a second reported surface that could induce Nef dimerization, composed of amino acids F139, R188, F191, H192, H193, R196, and E197 (4).

It is clear that some residues in the Nef protein are not readily analyzable. Unconserved residues like R105 give minimally disruptive phenotypes when mutated to A (Fig. 2). Highly conserved residues like D123 give multiple defects when mutated to the chemically related residue E (Fig. 2). The reality is that the structure of the Nef protein is just too complicated to assign specific functional interactions to a given residue without extensive data to support that assignment. It is also important to mutate multiple residues to determine which residue is the most informative. Within the context of subtype B Nefs, F191 is an amino acid to which we tentatively assign the disruption of a specific function. We propose that F191 mutations should be employed for investigating the role of PK-2 activation in newly described Nef functions and not R106 mutations (20, 48, 50, 55). D123E can serve as a positive control for this analysis, since it is defective for three major Nef functions but not PK-2 activation (Fig. 2).

Our determination that the SF2Nef mutants F191R and F191Y were negative for PK-2 activation while noting that subtype E Nefs have an R at position 191 framed the initial questions for subsequent investigation. The location of F191 focused our attention to the opposite side of Nef from the dimerization region (4, 21, 35). Upon examination of the refined three-dimensional structure of Nef (24, 39), it is evident that positions 85 and 89 are close to the putative SH3 binding domain (see Fig. S1, bottom, in the supplemental material). Thus, the concerted action of these two Nef regions in bringing about PK-2 activation remains a viable, albeit unproven, possibility. Unfortunately, the pathological significance of PK-2 activation by Nef remains to be determined, because biochemical evidence identifying the components of the Nef/PK-2 complex remains elusive. The major difficulty is the low abundance of the Nef/PK-2 protein complex (47). Our identification of the C-terminal region of Nef as critical for PK-2 activation might explain why others have not found PK-2 coinmunoprecipitating with C-terminal-tagged Nef, due to steric hindrance (27).

The discovery of different sets of covarying amino acids in Nef proteins capable of activating PK-2 suggests an important role for this function in HIV-1 replication and/or transmission. Though the pathological significance of the activation of PK-2 by Nef has been questioned (33, 44), we find that view difficult to reconcile with our mutational analysis. The data presented in this report suggest that Nef proteins with alternative structural motifs for the activation of PK-2 can be stable entities within HIV-1 group M subtypes. On the other hand, the limited number of subtype B Nefs containing F191R suggests a low level of shifting within a subtype. These results highlight our current lack of understanding of the complexities of Nef structure, function, and evolution. Unfortunately, the existence of alternative sets of amino acids that affect Nef function is a potentially significant obstacle for developing antiviral drugs based on Nef, particularly if this scenario applies to additional Nef functions. This potential complication added to the already bleak prospect for Nef-based antivirals (23) emphasizes the need for rigorous structure-function investigations guided by the information present in Nef amino acid sequence databases.

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