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Allosteric Loss-of-function Mutations in HIV-1 Nef from a Long-Term Non-Progressor

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Abstract

Activation of Src family kinases by HIV-1 Nef may play an important role in the pathogenesis of HIV/AIDS. Here we investigated whether diverse Nef sequences universally activate Hck, a Src family member expressed in macrophages and other HIV-1 target cells. In general, we observed that Hck activation is a highly conserved Nef function. However, we identified an unusual Nef variant from an HIV-positive individual that did not develop AIDS which failed to activate Hck despite the presence of conserved residues linked to Hck SH3 domain binding and kinase activation. Amino acid sequence alignment with active Nef proteins revealed differences in regions not previously implicated in Hck activation, including a large internal flexible loop absent from available Nef structures. Substitution of these residues in active Nef compromised Hck activation without affecting SH3 domain binding. These findings show that residues at a distance from the SH3 domain binding site allosterically influence Nef interactions with a key effector protein linked to AIDS progression.

Introduction

Nef is a small non-catalytic HIV-1 accessory protein essential for the pathogenesis of AIDS.^{1–5} Although some functions of Nef remain controversial, it is required for the high-titer replication of both HIV and SIV and is essential for the development of AIDS-like disease in Rhesus monkeys.⁵ HIV strains with defective *nef* alleles have been isolated from long-term non-progressors (LTNPs), implicating Nef as a critical virulence factor for AIDS.^{6–8} Furthermore, targeted expression of Nef in the T-cell and macrophage compartments of transgenic mice induces a severe AIDS-like syndrome, illustrating an essential role in disease progression.⁹

Non-receptor protein-tyrosine kinases of the Src family have been identified as key host cell effector proteins for HIV-1 Nef. For example, HIV infection induces Nef-dependent activation of the Src-family kinase (SFK) Hck in brain-derived microglial cells, and replication is blocked

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by co-expression of CD45 phosphatase or dominant-negative Hck.¹⁰ Similarly, suppression of Hck expression with antisense oligonucleotides dramatically inhibits M-tropic HIV replication in primary human macrophages, directly supporting a role for Hck in viral replication.¹¹ At the whole animal level, transgenic mice expressing Nef mutants unable to bind to the SH3 domain of Hck or other SFKs fail to develop an AIDS-like syndrome.¹² In addition, Nef-induced AIDS-like disease is delayed in mice lacking both *Hck* alleles, providing evidence that interaction of Nef with Hck and other SFKs may contribute to AIDS progression.¹²

Most studies of Nef:SFK interaction involve a handful of well-characterized laboratory isolates of HIV-1 Nef.^{13–16} This led us to question whether SFK activation, particularly of Hck, was a general function of HIV-1 Nef variants from a wider spectrum of both laboratory and patient-derived alleles. In the present study, we investigated this question using a yeast-based screen as well as in vitro kinase and SH3 binding assays with purified recombinant components. All of the Nef alleles used here encode the conserved PxxP motif and hydrophobic pocket residues essential for SH3 binding, allowing an investigation of other regions of the protein that may be involved in SFK activation. We found that common laboratory Nef alleles strongly activated Hck in our yeast assay in a manner consistent with previous results in mammalian cells.¹⁵ However, analysis of primary HIV Nef clones from LTNP identified an unusual allele that failed to activate Hck despite the presence of the PxxP motif and hydrophobic pocket. Sequence alignments and molecular modeling revealed amino acid differences at a distance from the SH3 domain binding site, with one of these residues mapping to a long internal flexible loop not present in the X-ray crystal or NMR structures of Nef.^{17,18} Substitution of these residues in Nef-SF2, a laboratory allele that strongly activates Hck, led to a significant loss of function in terms of kinase activation without affecting SH3 binding. These results suggest for the first time that Src-family kinase activation by Nef is under allosteric control of more flexible regions of the protein at a distance from the SH3-binding site.

Results

Activation of Hck is a conserved feature of laboratory HIV-1 Nef alleles

Previous work from our laboratory and others has established that HIV-1 Nef interacts with Hck through its SH3 domain, leading to constitutive kinase activation in vitro and in mammalian cells.^{13–16,19} These previous studies were conducted with a small sample of HIV-1 Nef alleles from laboratory strains of HIV-1, leaving open the question of whether SFK activation is a common feature of Nef sequences derived from a wider spectrum of HIV-1 strains. To answer this question, we turned to a yeast-based model system, recently applied in our group to the analysis of Nef:SFK interaction.¹⁶ SFKs are active following ectopic expression in *S. cerevisiae*, leading to growth suppression and increased tyrosine phosphorylation of yeast cell proteins.^{16,20,21} SFK activity is inhibited by co-expression of the negative regulatory kinase Csk, restoring yeast growth and diminishing SFK-mediated phosphorylation of yeast proteins. Csk phosphorylates SFK C-terminal tail tyrosine residues and induces intramolecular binding to SH2, which is required for efficient downregulation of SFK activity. In this way, SFK-induced alterations in yeast growth provide a simple read-out of relative kinase activity.

The effects of Csk and Hck on yeast growth are shown in Figure 1A. Yeast cultures were transformed with galactose-inducible expression plasmids for Csk and Hck, either alone or in combination. Transformed cells were then spotted as a series of dilutions on galactose-agar plates and incubated for several days. Expression of Csk alone had no effect on yeast growth. In contrast, Hck expression produced marked growth suppression, which is clearly evident at the 16-fold dilution. This effect of Hck is reversed by co-expression of Csk, consistent with our recent report.¹⁶ The effects of Hck expression on growth also correlate with protein-

tyrosine phosphorylation of endogenous yeast cell proteins¹⁶ (data not shown). Control immunoblots confirmed expression of Hck and Csk.

We next evaluated the ability of Nef alleles from six laboratory HIV-1 strains (Consensus, ELI, LAI, NL4-3, SF2, and YU-2) to activate Hck in the presence of Csk. As shown in Figure 1A, the Consensus, LAI, NL4-3, SF2, and YU-2 Nef proteins all induced strong growth suppression when co-expressed with Hck and Csk in comparison to Hck and Csk alone. This Nef-induced growth suppression correlated with markedly increased tyrosine phosphorylation of yeast proteins (data not shown). In contrast, the Nef-ELI protein had no effect on yeast growth when co-expressed with Csk and Hck, and failed to increase Hck kinase activity. This result is consistent with our previous findings in mammalian cells, and can be attributed to replacement of ELI Tyr-120 with Ile in the hydrophobic pocket required for Hck SH3 domain binding.¹⁵ None of these Nef alleles affected yeast growth when expressed in the absence of Hck (data not shown).

In order to simplify our assay, we tested the ability of the laboratory Nef alleles to activate a Hck variant capable of Csk-independent downregulation. Here the wild-type Hck C-terminal tail sequence is substituted with Tyr-Glu-Glu-Ile (Hck-YEEI), resulting in tail autophosphorylation and SH2 engagement as shown previously by X-ray crystallography.^{22, 23} Expression of Hck-YEEI alone failed to induce growth suppression in yeast, indicative of Csk-independent downregulation (Figure 1B). However, co-expression of the laboratory Nef isoforms activated Hck-YEEI and led to growth arrest, with the exception of ELI as expected. These results agree with the data obtained using wild-type Hck and Csk (Fig. 1A) as well as previous work in mammalian cells¹⁴, indicating that Hck-YEEI is a good surrogate for Csk-downregulated Hck.

Identification of an LTNP Nef allele that fails to activate Hck

Using the yeast system, we next screened a panel of eight primary *nef* gene products for their effects on Hck function. These Nef alleles were derived from long-term non-progressors (LTNPs), individuals who are seropositive for HIV-1 infection for at least eight years with no declining CD4 counts in the absence of therapy and do not progress to AIDS. Nucleotide sequence analysis revealed no internal deletions or truncations, and the coding regions for the consensus PxxP motif and core hydrophobic pocket residues required for SFK SH3 domain binding are intact (Figure S1). Hck-YEEI was co-expressed with each of the LTNP Nef alleles, along with Nef-SF2 as a positive control and a Nef-SF2 PxxP to AxxA mutant (PA) as a negative control.^{13,19,24} Seven of the eight primary Nef proteins induced Hck-mediated growth arrest, indicative of Hck kinase activation (Figure 2A). Strikingly, one of the LTNP Nef proteins (LTNP4) failed to cooperate with Hck-YEEI to induce growth suppression, despite an intact PxxP motif and hydrophobic pocket. To provide an independent assessment of the effect of this primary Nef allele on Hck kinase activity, we turned to an in vitro kinase assay using recombinant purified proteins. As shown in Figure 2B, Nef-LTNP4 failed to activate Hck-YEEI, consistent with results from the yeast assay. Nef-SF2 induced strong activation of Hck under the same conditions, consistent with our previous results.¹⁶ Results from these two assays suggest that amino acid substitutions outside of the Nef SH3-binding surface affect LTNP Nef function in terms of SFK activation.

Amino acid sequence alignment (Figure S1) and molecular modeling (Figure 3A) revealed several candidate residues responsible for the unexpected loss of Nef-induced Hck activation. These include Ala-156, which maps to the internal flexible loop, and Phe-191, which localizes to the Nef core away from the SH3-binding site. The positions of these residues within a structural model of full-length Nef are shown in Figure 3A, and are replaced with Ile and Leu, respectively, in Nef-LTNP4. To determine whether these residues affect Nef-induced SFK activation, we introduced the Nef-LTNP4 substitutions at these positions within the Nef-SF2

coding sequence. The resulting proteins were expressed in bacteria, purified to homogeneity and tested for their ability to activate Hck using the in vitro kinase assay. As shown in Figure 3B, substitution of either Ala-156 with Ile (A156I) or Phe-191 with Leu (F191L) significantly diminished Hck activation by Nef-SF2. Combining both of these substitutions in Nef-SF2 reduced Hck activation even further, reminiscent of the low activity of Nef-LTNP4 itself (Figure 2).

Analysis of Nef:SH3 interaction by hydrogen exchange mass spectrometry

To determine whether these LTNP-associated mutations affect Nef binding to the Hck SH3 domain, we turned to an assay based on hydrogen exchange mass spectrometry (HXMS).^{25–28} This assay takes advantage of the inherent conformational dynamics of the Hck SH3 domain, which is quite flexible in solution and partially unfolds with a half-life of approximately 15 minutes.²⁵ Unfolding is readily observed during the course of deuterium labeling as the presence of a bimodal distribution in the protein mass spectrum (Figure 4A). In the presence of high-affinity ligands, unfolding is dramatically slowed^{25–27}, providing a sensitive, solution-based assay for SH3:ligand interaction.

Using the HXMS assay, we tested whether the LTNP Nef mutations that interfered with Hck activation (Figure 3) affected Hck SH3 domain binding. Purified recombinant wild-type Nef-SF2, as well as the A156I, F191L and double (A156I/F191L) mutants, were incubated with purified Hck SH3 protein followed by HXMS analysis. Recombinant Nef-ELI, which does not bind or activate Hck, had no effect on SH3 unfolding and served as a negative control (Figure 4B). On the other hand, wild-type Nef-SF2 almost completely quenched the unfolding of the Hck SH3 domain, consistent with high-affinity binding (Figure 4C). Remarkably, all three mutant forms of Nef-SF2 bound to the Hck SH3 domain as strongly as wild-type (Figure 4E) despite their compromised ability to stimulate Hck kinase activity (Figure 3). This observation reveals for the first time that SH3 binding alone is necessary but not sufficient for Nef-induced Hck activation and that residues at a distance from the SH3-binding surface can influence kinase activation.

We also investigated SH3 binding by Nef-LTNP4, which was unable to activate Hck either in yeast or in vitro (Figure 2). As shown in Figure 4, Nef-LTNP4 slowed Hck SH3 domain unfolding by about 5-fold relative to the non-binding Nef-ELI negative control. These results indicate that additional amino acid differences relative to Nef-SF2 affect both SH3 domain binding as well as kinase activation, despite absolute conservation of known motifs involved in SH3 contact. Alignment of Nef-LTNP4 with Nef sequences known to activate Hck reveals only a handful of non-conservative substitutions unique to the LTNP4 sequence (Figure S2). Two of these map to the flexible N-terminal region, which has recently been suggested to interact with the core and may influence effector protein binding.²⁹ The other residues are Tyr-40, which is found in other LTNP alleles that retain Hck activation, and Gly-63, which is located in a conserved acidic stretch important for MHC downregulation.^{30,31} None of these residues is positioned to influence SH3 binding directly, providing support for the idea that seemingly subtle changes in the Nef amino acid sequence can have a profound impact on its ability to recruit and activate SFKs by an allosteric mechanism (see Discussion).

Discussion

Protein-tyrosine kinases of the Src family have emerged as important cellular signaling partners for HIV-1 Nef. Nef interacts with SFKs through their SH3 domains, leading to kinase activation that may be important for both HIV-1 replication and AIDS progression (see Introduction). Most molecular and structural analyses of Nef:SFK interactions have been limited to Nef alleles from laboratory HIV strains, such as SF2 and NL4-3. While the key structural motifs required for SH3 domain engagement and SFK activation have been defined, sequence differences

within the unstructured regions of Nef raise the possibility of allelic variation among primary Nef alleles in terms of SFK activation. Recently, we described a yeast-based assay which faithfully reconstitutes Nef:SFK interaction, leading to constitutive kinase activation that produces a growth-inhibitory effect that is easily scored.¹⁶ In the present report, we evaluated a wide variety of Nef proteins for their ability to activate the key SFK effector Hck using this system. We first validated the assay using the laboratory Nef alleles Consensus, ELI, LAI, NL4-3, SF2, and YU2. All of these Nef proteins potently activated Hck with the exception of Nef-ELI, previously shown to lack a hydrophobic pocket residue essential for SH3 domain binding and kinase activation.¹⁵ Each of the functional Nef proteins activated both wild-type Hck that was repressed by co-expression of the negative regulatory kinase Csk as well as a modified form of Hck which undergoes auto-downregulation by phosphorylating its own tail (Hck-YEEI). These results agree with our previous work in mammalian cells,¹⁵ providing validation for the yeast system as a screening tool to evaluate allelic variants of Nef for their impact on SFK activity.

We next used the yeast assay to screen a panel of patient-derived Nef alleles from LTNP, HIV-positive individuals who have survived for long periods without symptoms of AIDS. Nucleotide sequence analysis of the LTNP Nef alleles used here showed that they were full-length and possessed the known structural determinants of high-affinity Hck SH3 domain binding and kinase activation (PxxP motif and hydrophobic pocket; Figure S1). Despite the presence of these conserved structural features, the yeast assay identified one LTNP allele that failed to activate Hck (Nef-LTNP4). Follow-up experiments showed that recombinant purified Nef-LTNP4 was a very poor activator of Hck *in vitro*, suggesting that previously unrecognized structural features of Nef may contribute to SFK activation. Alignment of the Nef-LTNP4 sequence with that of Nef-SF2 and other strong SFK activators (Figure S2) revealed several substitutions, most notably Ile for Ala-156, and Leu for Phe-191. Neither of these residues is positioned for direct SFK SH3 domain binding as predicted from the Nef:SH3 co-crystal structure¹⁸ (Figure 3). Phe-191 localizes to the structured Nef core on the face opposite to the SH3 binding surface, while Ala-156 maps to a large flexible loop which is not present in existing Nef structures^{17,32}. Substitution of either residue in the context of Nef-SF2 substantially reduced its ability to activate Hck, raising the intriguing possibility that conformationally unrestricted Nef regions are essential for optimal kinase activation. This idea is supported by our surprising observation that the Nef-SF2 A156L and F191L mutants retain SH3-binding activity equivalent to wild-type Nef (Figure 4), despite reduced kinase-activating function (Figure 3). An additional possibility is that these flexible regions of Nef contact other SFK regions important for kinase activation.

Sequence variations found in Nef-LTNP4 that cause loss of SFK activation are relatively uncommon. O'Neill et al.³³ recently aligned 1,643 primary Nef sequences from HIV-1 clade B, the most common HIV-1 subgroup in North America. Substitution of Ala-156 with Ile occurred in only 0.4% of the cases, while substitution of Phe-191 with Leu was slightly more common, occurring 2.5% of the time. This study also found that Phe-191 is replaced with other residues in HIV-1 subtypes E (Arg) and C (His), suggesting that Nef proteins from these subtypes may show diminished activity towards Hck or other SFKs. Interestingly, Phe-191 has also been linked to the activation of the Pak2 kinase, suggesting a more general role for the Nef structural features controlled by this residue in kinase activation pathways.³³ Our finding that residues at a distance from the SH3-binding site can have a profound impact on SFK recruitment and activation strongly supports the idea that Nef is a highly plastic, dynamic structure.³⁴ Small molecules that restrict Nef conformational freedom may interfere with SFK recruitment and activation, providing a new approach to anti-HIV therapy.

Materials and Methods

Yeast constructs

Coding sequences for human Csk and Hck were amplified by PCR from existing templates to introduce a yeast translation initiation sequence (AATA) immediately 5' to the ATG start codon. Nef cDNAs from laboratory HIV-1 strains as well as primary LTNP were isolated as described elsewhere.^{15,35} The cDNA clones for HIV-1 Nef alleles were similarly amplified and modified for yeast expression. A FLAG tag was added to the C-terminus of each Nef coding sequence to provide a conserved epitope for immunoblotting. Hck was subcloned downstream of the Gal10 promoter in the pYC2/CT vector (Invitrogen), which carries the CEN6/ARSH4 sequence for low-copy replication. The Csk and Nef cDNAs were subcloned downstream of either the Gal 1 or Gal10 promoter in pESC-Trp (Stratagene). The Nef-2PA mutant, in which prolines 72 and 75 are replaced with alanines, has been described elsewhere.¹³

Yeast growth suppression assay

Yeast culture, transformation and growth suppression assay are described elsewhere.¹⁶ Briefly, cultures of *S. cerevisiae* strain YPH 499 (Stratagene) were electroporated with expression plasmids and selected on synthetic-dropout medium lacking uracil and tryptophan (SD/-Ura/-Trp) for three days at 30 °C on glucose agar plates. Positive transformants were grown in liquid selection medium containing glucose, normalized to OD₆₀₀ = 0.2 in water, and then spotted as a dilution series on SD/-Ura/-Trp agar plates containing galactose as the sole carbon source to induce protein expression. Plates were incubated for three days at 30 °C and imaged on a flatbed scanner, where yeast patches appear as dark spots against the translucent agar background. All growth suppression assays were repeated at least three times from independent transformed colonies of which representative examples are shown.

Immunoblotting

Liquid yeast cultures used for the spot assay were incubated in SD/-Ura/-Trp medium plus galactose for 18 h. Cells were pelleted, treated with 0.1 N NaOH for 5 min at room temperature,³⁶ and resuspended with SDS-PAGE sample buffer to 0.02 OD₆₀₀ units per µl. Lysates (0.2 OD₆₀₀ units) were separated via SDS-PAGE, transferred to PVDF membranes, and probed with antibodies to Csk (C-20; Santa Cruz), FLAG (M2; Sigma), and Hck (N-30; Santa Cruz).

Expression and purification of recombinant proteins

Nef-LTNP4 was expressed in Sf9 insect cells and purified as described previously for Nef-SF2.¹⁶ Briefly, a hexahistidine tag was added to the N-terminus by PCR and the cDNA subcloned into the baculovirus transfer vector, pVL1392. A recombinant baculovirus was prepared by transfecting Sf9 insect cells with the transfer vector and Baculogold DNA according to the manufacturer's protocol (BD-Pharmingen). Recombinant SF2 and LTNP4 Nef proteins were purified using immobilized metal affinity chromatography. Purity and concentration were confirmed by SDS-PAGE and densitometry.

Nef-SF2 mutants A156I, F191L, and the double mutant AI-FL were created using standard PCR-based mutagenesis techniques and subcloned into the bacterial expression vector pET-14b (BD Biosciences - Novagen). Wild-type Nef-SF2 was also cloned into this vector and all four Nef proteins were expressed in *E. coli* strain BL21(DE3)pLysS with N-terminal hexahistidine tags. Soluble Nef proteins were purified by immobilized metal affinity chromatography; purity and concentration were assessed as described above for the Nef proteins expressed in insect cells. The human Hck SH3 domain was also expressed and purified using the pET system in *E. coli* as described previously.^{27,28}

In vitro kinase assay

Tyrosine kinase assays were performed using the FRET-based Z'-lyte kinase assay system (Invitrogen) as described elsewhere.¹⁶

Hydrogen-deuterium exchange mass spectrometry

HXMS analysis was performed as described elsewhere³⁷ with the following modifications. Recombinant Hck SH3 and individual Nef proteins were equilibrated together at 4 °C for at least 110 minutes before the initiation of the labeling reaction. Initial reactions consisted of 12 μM SH3 protein and 4.8 μM Nef protein in 20 mM Tris-HCl pH 8.3, 100 mM NaCl, and 3 mM DTT. Deuterium labeling was initiated by 15-fold dilution of the binding reaction into D₂O labeling buffer (20 mM Tris, pH 8.3, 100 mM NaCl, 3 mM DTT). Under these conditions, 89% of SH3 was estimated to be Nef-bound during labeling based on a K_D of 0.25 μM.³⁸ Aliquots of the reaction were quenched at various times by addition of 250 mM potassium phosphate, pH 2.6.

Labeled proteins were injected onto a POROS 20 R2 protein trap and desalted with 0.05% TFA at a flow rate of 500 μl/min. The proteins were eluted into the mass spectrometer using a linear 15–75% acetonitrile gradient over 4 min at 50 μl/min with a Shimadzu HPLC system (LC-10ADvp). HPLC was performed using protiated solvents which results in the removal of deuterium from the side chains and the amino/carboxy termini that exchange faster than backbone amide hydrogens.^{39,40} Mass spectral analyses were carried out with a Waters LCT-Premier^{XE} mass spectrometer with a standard electrospray source, a capillary voltage of 3.0 kV and a cone voltage of 35 V. The deuterium levels were not corrected for back-exchange^{39,40} and reflect relative changes across the protein samples. The relative deuterium incorporation for each SH3 domain was determined by subtracting the mass of the unlabeled SH3 domain from the mass of the SH3 domain at each time point. The isotope envelopes in bimodal patterns were determined by fitting the data with two Gaussian functions whose widths were estimated from the width of a single binomial isotopic envelope before and after the appearance of the bimodal pattern. The unfolding rates for those SH3 domains that presented evidence of a bimodal isotopic envelope were determined from the slope of pseudo-first-order kinetic plots of the decrease in the relative intensity of the lower mass envelope with time.^{25–27}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Yeast-based screen for HIV-1 Nef-induced Hck activation. A) Yeast cultures were co-transformed with expression constructs for Hck and Csk together with Consensus, ELI, LAI, NL4-3, SF2, or YU-2 Nef alleles. *Top*: Liquid cultures were normalized for cell density and spotted on galactose-agar plates at increasing dilutions, incubated for 3 days at 30 °C, and scanned. Yeast patches appear as dark circles against the translucent agar background.

Bottom: Lysates from the same cultures were immunoblotted for Hck, Csk, or Nef expression as indicated. B) Hck-YEEI replaces Hck plus Csk co-expression in the yeast assay. Yeast cultures were co-transformed with expression constructs for Hck-YEEI and Consensus, ELI, LAI, NL4-3, SF2, or YU-2 Nef alleles. *Top*: Yeast cultures were grown on galactose-agar plates as

described in part A. *Bottom*: Lysates from the same cultures were immunoblotted for Hck or Nef as indicated.

Figure 2.

Activation of Hck-YEEI by primary Nef alleles from LTNPs. A) Yeast assay. Yeast cultures were transformed with expression constructs for Hck-YEEI alone (-Nef) or together with Nef-SF2 as a positive control or the LTNP Nef alleles indicated. A Nef-SF2 PxxP to AxxA mutant (PA) which cannot bind or activate Hck-YEEI served as a negative control. *Top*: Liquid cultures were normalized for cell density and spotted on galactose-agar plates at increasing dilutions, incubated for 3 days at 30 °C, and scanned. Yeast patches appear as dark circles against the translucent agar background. *Bottom*: Lysates from the same cultures were separated via SDS-PAGE and immunoblotted for Hck or Nef proteins as indicated. B) Nef-LTNP4 fails to activate Hck-YEEI in vitro. Recombinant Hck-YEEI was assayed in the presence of increasing molar ratios of purified recombinant Nef-SF2 or Nef-LTNP4 using a peptide substrate and a FRET-based kinase assay.¹⁶ Each condition was repeated in quadruplicate and the extent of phosphorylation is expressed as the mean percent phosphorylation relative to a control

phosphopeptide $\pm$ S.D. This experiment was repeated twice with comparable results; a representative example is shown.

Figure 3.

LTNP-derived amino acid substitutions outside of the SH3-binding surface suppress Hck activation by Nef-SF2 in vitro. A) Model of Nef:SH3 complex. The Nef backbone is shown in grey, and the SH3 surface is colored violet. The conserved PxxP motif and hydrophobic pocket residues of Nef involved in SFK SH3 binding are highlighted in cyan. Residues substituted in Nef-LTNP4 associated with loss of SFK activation are highlighted in red (F191) and green (A156). The full-length Nef model was originally created by Geyer and Peterlin;³² note that the large unstructured loop and flexible N-terminal region are not present in existing Nef:SH3 structures.^{17,18} B) Replacement of Nef-SF2 residues F191 and A156 with corresponding residues of Nef-LTNP4 reduces its Hck-activating function. Wild-type Nef-SF2

(WT) as well as the Nef-SF2 point mutants A156I, F191L and the corresponding double mutant (AI-FL) were expressed in *E. coli* and purified to homogeneity. Recombinant Hck-YEEI activity was assayed either alone or in the presence of a 5- or 10-fold molar excess of each Nef protein using a peptide substrate and FRET-based kinase assay.¹⁶ Each condition was repeated in quadruplicate and the extent of phosphorylation is expressed as the mean percent phosphorylation relative to a control phosphopeptide $\pm$ S.D. This experiment was repeated twice with comparable results.

Figure 4.

Determination of the relative binding affinity between Hck SH3 and Nef by HXMS. A–D) Recombinant purified Hck SH3 was labeled with deuterium for the periods indicated at the left of each panel. Changes in mass for the +5 charge state are shown for the Hck SH3 domain incubated alone (A) or in the presence of the non-binding control Nef-ELI (B), wild-type Nef-SF2 (C), or Nef-LTNP4 (D). Na⁺/K⁺ adducts are indicated with an asterisk. Labeling of SH3 in the presence of the Nef-SF2 mutants A156I, F191L and AI-FL gave identical results to those shown in panel C for wild-type Nef-SF2 (data not shown). Panel E shows the slowdown factor for the partial unfolding of the Hck SH3 domain either alone or in the presence of each Nef variant. The slowdown factor provides a relative measure of SH3 binding affinity for each Nef

protein and is calculated from the rate constants for SH3 unfolding in the presence and absence of Nef as described elsewhere.²⁷