Molecular Design, Functional Characterization and Structural Basis of a Protein Inhibitor Against the HIV-1 Pathogenicity Factor Nef

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Abstract

Increased spread of HIV-1 and rapid emergence of drug resistance warrants development of novel antiviral strategies. Nef, a critical viral pathogenicity factor that interacts with host cell factors but lacks enzymatic activity, is not targeted by current antiviral measures. Here we inhibit Nef function by simultaneously blocking several highly conserved protein interaction surfaces. This strategy, referred to as “wrapping Nef”, is based on structure-function analyses that led to the identification of four target sites: (i) SH3 domain interaction, (ii) interference with protein transport processes, (iii) CD4 binding and (iv) targeting to lipid membranes. Screening combinations of Nef-interacting domains, we developed a series of small Nef interacting proteins (NIs) composed of an SH3 domain optimized for binding to Nef, fused to a sequence motif of the CD4 cytoplasmic tail and combined with a prenylation signal for membrane association. NIs bind to Nef in the low nM affinity range, associate with Nef in human cells and specifically interfere with key biological activities of Nef. Structure determination of the Nef-Inhibitor complex reveals the molecular basis for binding specificity. These results establish Nef-NI interfaces as promising leads for the development of potent Nef inhibitors.

Introduction

Highly active antiretroviral therapy (HAART) currently employed to treat AIDS patients consists of a combination of drugs that target the HIV enzymes reverse transcriptase, protease and integrase as well as inhibitors of virus entry. Although HAART is highly effective, its high cost and limited availability in underdeveloped areas severely limit the success of current anti-HIV therapy. Moreover, the rapid emergence of drug resistance mutants and the increased worldwide spread of such treatment resistant HIV variants pose increasing problems to effective treatment of HIV-patients [1]. One of many strategies to improve this situation is the exploitation of additional drug targets that could be added to the current regimen. Ideally, such targets comprise viral factors, since interference with host cell factors often compromises physiological function or even viability of host cells [2].

The Nef protein is an accessory gene product of HIV and SIV that is dispensable for virus spread in experimental ex vivo cell culture systems [3]. In infected patients or monkeys however, Nef is critical for high virus replication and disease progression. In fact, defects in the nef gene lead to slow progressively or even asymptomatic infections and transgenic mice expressing Nef as the only HIV-1 gene product develop AIDS-like disease [4–7].

Thus, Nef is an important factor for AIDS pathogenesis. Although compounds interfering with Nef’s activity would thus be of obvious global benefit, Nef is currently not a target of antiviral measures. This lack of Nef targeting reflects the limited knowledge about the mechanism by which Nef promotes virus spread and accelerates disease progression in patients. Over the last years it has become clear that Nef’s impact on AIDS pathogenesis results from the combined action of several independent activities [8,9]. First, Nef acts as a factor that prevents recognition of HIV infected cells by the host immune system (immune evasion) via a reduction of the cell surface density of bioactive MHC class I and II molecules [10] and possibly by restriction target cell motility [11]. Second, Nef alters the activation state of HIV target cells to increase their permissivity to virus replication and prolongs their life span to optimize virus production [9,12,13]. Third, Nef augments the infectivity of HIV particles [14]. This effect is not potentiated over several rounds of replication due to efficient but Nef-insensitive cell-to-cell spread, however accounts for the slight delay in replication kinetics observed for nef-deficient HIV-1 [15]. To achieve this multitude of activities, Nef has evolved as versatile adaptor for protein interactions that lacks intrinsic enzymatic activity, which allows the viral protein to affect a wide range of host cell protein sorting and signal transduction processes [3]. The lack of enzymatic activity and of a well defined ex vivo assay system
that mirrors the complexity of Nef's biological activities in vivo, however, hampers the development of potent inhibitors that are effective against a broad range of Nef functions. Thus, the previously described Nef-interacting small compounds bind Nef only with relatively low affinity, display high cytotoxicity and/or interfere with only a subset of Nef interactions and functions [16–19]. We therefore reasoned that a rational and structure-based approach may be required for the development of an effective and multifunctional Nef inhibitor.

The structure of HIV-1 Nef is characterized by its flexible loop regions that together comprise more than half of the protein sequence [20]. Nef contains an N-terminal myristoylation site followed by an amphiphatic helix for the association with cellular membranes that is thought to be critical for all Nef activities. A central PxxP motif for the interaction with SH3 domains is located within a loop section that bridges the N-terminal anchor domain to the structured core domain. This motif is essential for many interactions of Nef with host cell signal transduction cascades as well as for the downmodulation of cell surface receptors such as MHC-I and CCR5 [21,22] and overexpression of isolated SH3 domains that bind to Nef with high affinity can interfere with its activities [23]. A dileucine based endocytosis motif (ExxxLL) is exposed at the tip of an approximately 30 residue encompassing C-terminal flexible loop to mediate the interaction with protein transport complexes, an interaction that mediates cell surface removal of CD4 and enhancement of virion infectivity by Nef [24,25]. The recognition site for the cytoplasmic tail sequence of CD4 instead is supposed to reside within a hydrophobic groove on the core domain structure of Nef [26].

The assembly of such diverse yet highly conserved interaction motifs spread out in various flexible loop sections of the target protein renders the generation of Nef specific inhibitors that interfere with more than one individual Nef function at a time a challenging task. We thus chose a strategy aimed at wrapping the surface of Nef by the combination of different, physically linked, target domains and the iterative optimization of domain composition and linker segments.

Results

Molecular design of HIV-1 Nef interacting proteins

Nef is composed of a series of interaction motifs that spread over the surface of the protein in flexible regions at diverse sites and mediate specific interactions with host cell proteins (Figure 1A). Nef mutagenesis studies established that only simultaneous disruption of several independent protein interactions of Nef efficiently abrogates the complex array of its biological activities [8]. We therefore hypothesized that a Nef protein inhibitor has to shield several of these interaction motifs at a time. In addition, targeting multiple conserved regions simultaneously will limit the ability of resistance development. Based on the currently available knowledge about the structure-functional relationship in HIV-1 Nef, several candidate interaction motifs were considered (Figure 1B). First, we selected the SH3 domain of Hck as lead structure (referred to as ‘Nef-Interacting protein 1-1’ or ‘NI1-1’) to target the central PxxP motif in the poly-proline rich loop of Nef because of its relatively high affinity binding to Nef [27]. Variations of six residues in the so-called RT-loop in between the first and second β-strand of the SH3 domain further increase the affinity to Nef by 20- to 30-fold [28]. Besides human wild type Hck-SH3 (78–138) that contained the sequence E90AIHHE within the RT-loop, we therefore also generated the two mutant SH3 domains V90SWSPD and Y90SPFSW, termed NI1-2 and NI1-3, respectively.

Second, we included a helical region in the β-subunit of the adaptor protein complex 2 (AP-2) that was supposed to act as binding site for dileucine based sorting motifs [29]. Constructs encompassing aa 279–310 or 352–321, corresponding to either eight or six HEAT repeats of the domain structure, respectively, were generated (series NI2). Third, we used the cytoplasmic tail sequence of CD4 itself to interact with its recognition site on Nef. Two different lengths of this peptide (37 or 23 residues) were employed either as wild type sequence or in conjunction with mutation of an Lck-binding motif CQC to SQS, or mutation of the dileucine based internalization motif LL to AA (series NI3). As alternative approach to target the dileucine internalization motif in Nef, the VHS domain of the human GGA2 protein (residues 21–164) was used that was shown to bind acidic-cluster-dileucine sorting signals of the mannose-6-phosphate receptor [30,31]. Finally, for those constructs that drive NI expression in human cells, we added a lipidation signal either as N-terminal myristoylation motif (MGxXXS) or as C-terminal farnesylation motif (CVLS) to the protein, sometimes in combination with additional palmitoylation sites, for the targeting to cellular membranes. Likewise, an HA-epitope was added always at the alternate site of lipidation in the cellular expression constructs for antibody recognition.

The selected sequences were fused in various combinations using different linker length and variable domain successions to result in four different generations of putative Nef-interacting molecules (Figure 1C). These fusion proteins were designed to interact simultaneously with multiple binding sites of Nef, leading thus to increased affinity and specificity for the viral protein. In total 23 different constructs were designed and expressed for in vitro studies to characterize their binding affinities to Nef and another 25 constructs for studies in human cells. An overview of the constructs generated is shown in Figure S1.

Binding specificity between Nef and inhibitor proteins

We first purified the recombinant NI proteins (Figure 1D) and analyzed their binding capacities to HIV-1 Nef in vitro. Binding affinities of the direct interactions were determined by isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) or fluorescence spectroscopy to compare the dissociation constants of the various fusion proteins. Nef (Δ1-44) bound to the wild type Hck-SH3 domain (termed ‘NI1-1’) with a $K_d$ value of 1.54 μM in agreement with previous studies [32]. Mutation of the RT-loop sequence E90AIHHE to VSWSPD (‘NI1-2’) or YSPFSW (‘NI1-3’) led to an increase of the Nef binding affinity to 179 nM or even 48 nM, respectively, indicating the enormous contribution of this region to the PxxP recognition of the SH3 domain (Figure 2A and Table 1). To preserve the experimental range of interaction for further improvements we used in the following the VSWSPD sequence in the SH3 domain as a lead structure in the next generations of fusion proteins.

Fusion of the proposed dileucine binding domains to the SH3 domain (generations NI2 and NI4) resulted in protein products that tended to precipitate upon purification in vitro. Likewise, these constructs were unstable when expressed in mammalian cells (data not shown) and could therefore not be investigated further. Attachment of the cytoplasmic domain of CD4, however, which is the smallest unit tested, turned out to be suitable for in vitro characterization and amenable for structure-function based binding improvements. N-terminal fusion of CD4 to SH3 by a five residue linker resulted in a dissociation constant of 84 nM of the 103 residue encompassing protein (NI3-1) and shortening of the CD4 segment to 23 residues similarly resulted in a $K_d$ of 107 nM (NI3-8). Importantly, mutation of two cysteines (CQC to
constant between His-tagged CD4-SH3 (NI3-9) and four different surface plasmon resonance was used to determine the dissociation concentrations of wild type full length Nef (Figure S2). Similarly, that contained only one cysteine for labeling, with increasing
teriments using the dansyl labeled SH3-CD4 fusion variant NI3-3,
were confirmed by equilibrium fluorescence titration measure-
residues outside the core domain of Nef could still contribute to the
an amphipathic helix to sustain membrane binding [34], while
terminal region is embedded in the lipid bilayer where it may form
the N-terminal 27 residues only marginally affected the interac-
dissociation constant to NI3-13 of 41 nM was determined, its
proteins (Figure 2B). Together these results indicate that Nef associates
with NI3-1 and NI3-7 by co-immunoprecipitation analysis
that were subjected to further in depth analysis. Notably, the
localization of Nef.GFP was altered in the presence of these
constructs: while located at the plasma membrane, the cytoplasm,
as well as associated with cytoplasmatic membrane vesicles when
expressed alone, Nef.GFP was enriched at the plasma membrane
in the presence of NI3-1 and at intracellular vesicles upon co-
expression with NI3-7 and NI3-9. Most importantly and
pronounced for constructs NI3-1, NI3-7 and NI3-9 (Figure 3A)
localization of both proteins in most cases. This was most
revealed that, when expressed alone or together with a GFP
viral protein when expressed in cells. Based on the above in vitro
analysis and their small size we focused on the 3rd generation of
NIs. All constructs NI3-1 to NI3-14 resulted in the expression of
stable proteins to comparable levels in 293T cells (Figure S5).
Expression of these NIs, either alone or in combination with Nef,
did not result in apparent cytotoxicity. Confocal microscopy
revealed that, when expressed alone or together with a GFP
control, most NIs displayed a predominantly diffuse cytoplasmic
subcellular distribution with some localization to the plasma
membrane (Figure S5A). NI3-7, NI3-9 and NI3-11, however,
localized prominently to intracellular membrane structures.
Co-expression of Nef.GFP with the NIs resulted in marked
colocalization of both proteins in most cases. This was most
pronounced for constructs NI3-1, NI3-7 and NI3-9 (Figure 3A)
that were subjected to further in depth analysis. Notably, the
localization of Nef.GFP was altered in the presence of these
constructs: while located at the plasma membrane, the cytoplasm,
as well as associated with cytoplasmatic membrane vesicles when
expressed alone, Nef.GFP was enriched at the plasma membrane
in the presence of NI3-1 and at intracellular vesicles upon co-
expression with NI3-7 and NI3-9. Most importantly and
irrespective of the specific subcellular localization, high degrees of
colocalization were observed between the NIs and Nef.GFP (see
arrows). Consistently, Nef.GFP was found to physically associate
with NI3-1 and NI3-7 by co-immunoprecipitation analysis
(Figure 3B). Together these results indicate that Nef associates
with the NIs in intact human cells.

NIs interfere with biological activities of Nef
To address whether the association of NIs affects the biological
properties of Nef, a series of functional assays was performed in the
absence or presence of NI3-1, NI3-7 and NI3-9. The ability of
Nef.GFP to reduce cell surface presentation of the receptor
molecules CD4, CCR5, MHC-I and CD71 was evaluated by flow
cytometry (Figure 4A). While CD71 was included as a Nef-
insensitive negative control, downregulation of cell surface CD4,
MHC-I and CCR5 receptors are established Nef activities that
depend on select motifs in Nef. Nef-mediated downregulation of
CD4 requires endocytic motifs in the C-terminal flexible loop of
Nef as well as an interaction with the cytoplasmic tail of CD4 [3].
In contrast, effects of Nef on MHC-I and CCR5 are flexible loop
independent but require several interaction motifs in the Nef core
domain [21,22]. Expression of the NIs alone had no significant
effect on cell surface levels of CD4, CCR5, MHC-I or CD71 at
these levels of expression. In contrast, Nef significantly decreased
cell surface exposure of CD4, CCR5 and MHC-I but not of the
negative control CD71. These effects were inhibited with variable

Figure 1. Schematic display of Nef Interacting (NI) protein design. (A) Cartoon of interaction motifs within the Nef structure. (B) Protein domains and lipidation signal sequences employed for the design of Nef interacting proteins. The number of different constructs generated and the proposed target sites are shown to the right. (C) Fusion design of four different NI generations. Different recognition domains were assembled in alternating orders and combined with various membrane association motifs to result in different expression constructs, used either for recombinant protein production or in vivo cell expression. (D) Display of the three high affinity binders designed to wrap the interaction surfaces of Nef. (E) SDS PAGE display of selected recombinant Nef and NI proteins used for in vitro binding analyses.
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potency by the NIs: NI3-1 and NI3-9 almost completely prevented CD4 and MHC-I downmodulation by Nef, while NI3-7 displayed only partial inhibition of Nef-mediated CD4 downregulation. All three inhibitors had intermediate effects on CCR5 downmodulation by Nef. Of note, cross-talk of inhibitors NI3-7 and NI3-9 with the SH3 domain binding defective Nef (PxxPxR to AxxAxA) mutant. The dissociation constant of 1.2 μM revealed the putative contribution of the dileucine motif of CD4 to the Nef interaction. The thermodynamic parameters and the dissociation constants of the interactions are listed in Table 1.

Figure 2. Isothermal titration calorimetric measurements between Nef and selected Nef interacting proteins. (A) Binding analyses between Nef and optimized GST-Hck-CD4 chimeras. Displayed are ITC measurements with N11-1 (Hck, upper left), N11-2 (Hck-RT loop optimized; upper-right), NI3-1 (CD4-Hck; lower left) and NI3-9 (Hck-CD4; lower right). (B) Contribution of the length of the N-terminal membrane anchor domain of Nef to the binding to NI3-13. (C) Binding between the prototypic Nef inhibitor NI3-9 (HckSH3-polyGly-CD4) and the SH3 domain binding defective Nef (PxxPxR to AxxAxA) mutant. The dissociation constant of 1.2 μM revealed the putative contribution of the dileucine motif of CD4 to the Nef interaction. The thermodynamic parameters and the dissociation constants of the interactions are listed in Table 1.

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Table 1. Thermodynamic parameters of Nef-inhibitor interactions.

| Interaction (cell/syringe) | T (°C) | ΔG (kcal/mol) | ΔH (kcal/mol) | TΔS (kcal/mol) | molar ratio | Kd (nM) |
|----------------------------|-------|---------------|---------------|---------------|------------|--------|
| Nef (Δ1-44)/NI1-1          | 25    | −7.93         | −4.50±0.09    | 3.43          | 0.97       | 1537±195 |
| Nef (Δ1-44)/NI1-2          | 25    | −9.20         | −8.16±0.07    | 1.04          | 0.99       | 179±29  |
| Nef (Δ1-44)/NI1-3          | 25    | −9.98         | −7.99±0.07    | 2.00          | 0.95       | 48±11   |
| Nef (wt)/NI1-1*            | 15    | −8.71         | −4.24±0.03    | 4.47          | 1.08       | 249±27  |
| Nef (wt)/NI1-2*            | 15    | −9.04         | −8.90±0.02    | 0.138         | 1.07       | 140±6.7 |
| Nef (wt)/NI1-3*            | 15    | −9.33         | −8.51±0.03    | 0.82          | 1.09       | 84±7.1  |
| Nef (wt)/NI3-8*            | 15    | −9.19         | −8.20±0.04    | 0.99          | 0.93       | 107±10.6|
| Nef (wt)/NI3-5*            | 15    | −9.49         | −8.22±0.04    | 1.27          | 1.06       | 64±7.5  |
| Nef (wt)/NI3-3*            | 15    | −9.30         | −8.64±0.02    | 0.66          | 1.12       | 88±5.4  |
| Nef (wt)/NI3-4*            | 15    | −8.26         | −8.55±0.07    | −0.288        | 0.97       | 544±59  |
| Nef (wt)/NI3-9*            | 15    | −10.01        | −11.40±0.03   | −1.39         | 0.97       | 26±2.1  |
| Nef (wt)/NI3-13*           | 15    | −9.74         | −7.04±0.03    | 2.70          | 0.98       | 41±4.4  |
| Nef (wt)/NI3-10*           | 15    | −9.98         | −11.50±0.03   | −1.52         | 1.03       | 27±1.8  |
| Nef (wt)/NI3-11*           | 15    | −9.25         | −6.91±0.02    | 2.35          | 1.10       | 97±5.0  |
| Nef (AxxA)/NI3-9*          | 25    | −8.09         | −4.10±0.05    | 4.00          | 0.85       | 1173±111|
| Nef (Δ1-27)/NI3-13*        | 15    | −9.61         | −12.22±0.10   | −2.61         | 1.00       | 51±9.8  |
| Nef (Δ1-44)/NI3-13*        | 15    | −9.26         | −16.13±0.13   | −6.87         | 1.06       | 96±15   |
| Nef (Δ1-58)/NI3-13*        | 25    | −9.31         | −13.81±0.18   | −4.50         | 0.98       | 149±27  |

*These samples were used as GST-fusion proteins.

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characteristic hydrophobic interface for interactions. The numbering of secondary structure elements is based on the assembly of a full length Nef structure composed of the N-terminal anchor and the C-terminal core domain [20]. The accessible surface of helices α4 and α5 is separated into two parts by the highly conserved residues F94 and W117, whose aromatic side chains oppose each other and act as gatekeepers to form a barrier in the helical region (Figure 5B). The mutant residues V90SWSPD of the Hck RT loop undergo tight interactions with the proximal region of these antiparallel helices, with tryptophan 92 of Hck contributing itself to the high binding affinity with 41 interactions to six different residues of Nef within a shell of 4 Å. Many of these interactions were mediated by stacking of the aromatic side chains W92Hck to F94Nef, as well as facing interactions to W117Nef. In addition, R81Nef in the PxxPxR motif of Nef showed direct ionic interactions to D96 of Hck and the side chain of Q122Nef made

Figure 3. Association of Nef with NIs in human cells. (A) Localization of Nef and NIs in HeLa cells. GFP or Nef.GFP was co-expressed with an empty control vector or the indicated NIs and subjected to confocal microscopy analysis following fixation and anti-HA immunostaining. Presented are confocal sections of the middle of representative cells. Scale bar = 10 μm. Arrows indicate examples of colocalization between Nef.GFP and NIs. (B) Co-immunoprecipitation of Nef.GFP and the indicated NIs from HeLa cells. Shown is a Western blot analysis of Nef.GFP and NIs in the input cell lysate (upper panel) and following anti-GFP immunoprecipitation (lower panel).

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tight interactions to the hydroxyl group of V90 Hck (2.9 Å). Likewise, the hydroxyl of the two countercurrent helices with W61 of Nef interacting with the main chain oxygen of Y90 Hck (2.9 Å). Likewise, the hydroxyl group of S93 Hck was hydrogen bonded to D90 Nef (2.6 Å) and D95 Hck formed an ionic interaction to K86 of Nef (Figure S6A,B).

While the tight interactions that account for the increased affinity of mutant Hck NI1-2 compared to wild type Hck occurred all at the hydrophobic site of the two helices in Nef proximal to the PxxP motif, we now observed a previously unresolved additional contact on this structure element.

Table 2. Crystallographic data collection and structural refinement statistics.

|                     | NefSF2 (45-210,158-178) – NI1-2 | NefSF2 (45-210,158-178) – NI3-13 |
|---------------------|-------------------------------|----------------------------------|
| **Data collection** |
| Space group        | P4_{1}2_{1}2                  | P6_{1}                           |
| Cell dimensions    | a, b, c (Å)                   | 65.69, 65.69, 279.07             | 112.46, 112.46, 130.03            |
| α, β, γ (%)        | 90, 90, 90                    | 90, 90, 120                      |
| Wavelength         |                               | 0.9                              | 0.9763                           |
| Resolution (Å)     | 50 – 2.0 (2.1 – 2.0)          | 20 – 3.45 (3.55 – 3.45)         |
| Rmerge             | 5.2 (27.5)                    | 6.6 (30.5)                      |
| (I/σ (I))          | 18.2 (4.4)                    | 16.6 (3.9)                     |
| Completeness (%)   | 100 (100)                     | 99.9 (100)                      |
| Redundancy         | 26.6 (23.4)                   | 12.6 (12.8)                     |
| **Refinement**     |
| Resolution (Å)     | 50 – 2.0 (2.05 – 2.0)         | 20 – 3.45 (3.71 – 3.45)         |
| No. reflections    | 40486 (3095)                  | 12282 (2442)                    |
| Rwork              | 16.8 (20.0)                   | 21.6 (28.0)                     |
| Rfree              | 20.5 (21.7)                   | 24.3 (35.5)                     |
| No. atoms          | Protein 3084                  | Water 2761                      |
|                    |                                | –                                |
| Factors            | Protein 39                    | 39                               |
|                    | Water 46                      | 132                              |
| R.m.s deviations   | Bond lengths (Å)              | 0.026                            | 0.016                            |
|                    | Bond angles (°)               | 1.823                            | 1.584                            |
| PDB entry code     | 3REA                          | 3REB                             |

1 All data sets were collected from one single crystal.
2 Values in parentheses refer to the highest resolution shell.

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The CD4 moiety interacts with the core domain structure of Nef

Crystals of Nef (45-210, Δ158-178) in complex with NI3-13 were grown under similar conditions but despite extensive testing by seeding, dehydration and additives, these crystals diffracted only to 3.5 Å resolution. Unfortunately, there was no electron density visible that would unambiguously account for the CD4 part of the inhibitory molecule. However, the resolution achieved was sufficient to show that the N-terminal chain of Nef in one complex of the asymmetric unit was bended away such that the binding site distal to the PxxP motif on face the N-terminus of the core domain of Nef (Figure 3B). This helix, ranging from residue A60 to E69, bound at the distal site of the two countercurrent helices with W61 of Nef interacting with hydrophobic residues L104 on the short β1 strand of Nef, I113, L114 and the methylene groups of R110 (Figure 5C). In the NMR structure of NefBH10 this tryptophan residue was previously reported to exist in two different conformations with one conformation attached to its core domain structure [26] (Figure S6C). Of note, the acidic cluster motif of four successive glutamates (E66–E69), that was reported to interact with the sorting adaptor protein PACT1 [38], adopts a helical conformation on this structure element.

Discussion

In this study we engineered a 10.0 kDa protein (36 amino acids) that binds to HIV-1 Nef in the low nano-molar range. This protein, NI3-13, targets three highly conserved sites in Nef, namely the PxxPxR loop, the RT loop recognition site on Nef and the binding site for the CD4 dileucine based sorting signals on Nef. For tissue culture experiments the inhibitor protein was addition-ally targeted to lipid membranes by a myristoylation/palmitoylation motif, which is thought to further increase the binding specificity to membrane-localized Nef. Thus, a protein construct was designed that simultaneously covered three interaction sites of
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Nef, wrapping its functional surface sites to render the protein non-functional.

The simultaneous targeting of multiple interaction sites is a well proven approach to generate high affinity and specificity binding to a protein of interest [39,40]. Indeed, we show here that the binding affinity of a designed protein inhibitor to HIV-1 Nef is increased from 250 to 26 nM by fusion of two structural elements and further lead optimization. This fusion protein is the tightest binder to Nef known to date and supposedly covers a large surface on the target protein. The selection of target sites within Nef was strictly dependent on high sequence conservation in HIV-1 Nef alleles of different subtypes and stages of disease. The ability to interact with the cytoplasmic tail sequence of CD4 at the plasma membrane is potentially the most conserved feature of Nef and an ancestral function in the evolution of this protein in HIV/SIV lentiviruses [11,41]. The binding of the central PxXP motif to cellular SH3 domains adds to the diversity of Nef function in signaling and trafficking aspects. The SH3 RT loop is known to interact with a unique hydrophobic groove on Nef that significantly contributes to the specificity of the viral protein in SH3 domain recognition [27,28]. Targeting of the inhibitor protein to cellular membranes by lipidation signals finally, further relates to the highly conserved presence of an N-terminal myristoylation signal in Nef and reduces the interaction space to a two dimensional layer.

Our functional studies in human cells provide proof-of-concept for potent inhibition of Nef function by the prototype HIV-1 Nef protein inhibitor. Importantly, this included inhibition of mechanistically independent activities such as down modulation of cell surface CD4 and MHC-I molecules, confirming the successful simultaneous interference with independent protein interactions of Nef in the absence of detectable cytotoxicity. Furthermore, inhibition of Nef was also achieved in the context of infectious HIV-1, where positive effects of Nef on the infectivity of viral progeny were blunted by the presence of the protein inhibitor. These results warrant the development of future generations of such prototype inhibitors. However, therapeutic exploitation of anti-infectives based on intracellular expression of protein inhibitors are hampered by difficulties including delivery as well as inhibitor stability and immunogenicity [42]. Similar to the recently generated inhibitors of interactions between HIV-1 integrase and its cellular cofactor LEDGF/p75 [43], such developments will build on the complex structure described here to rationally design small and cell-permeable Nef-interacting compounds. Their future functional characterization will focus on the full array of Nef activities, including analyses in primary human cells in the context of viral infection. Since one limitation of these analyses consists of the lack of potent effects of Nef on virus replication in cell culture systems, ultimate validation of Nef inhibitors will require investigations in in vivo models such as humanized mice or HIV-1 susceptible transgenic rats [44–46]. Irrespective of its potential future clinical application, motif-specific intracellular Nef inhibitors at present represent valuable tools for studies of host cell interactions of the viral pathogenicity factor.

The structures of Nef-NI1-2 and Nef-NI3-13 complexes described here reveal details of two conserved non-polar grooves in the HIV-1 Nef protein that are potential target sites for therapeutic intervention. Both sites are located on helices α4 and α5 of Nef that are spread apart by gatekeeper residues F94 and W117. While the site proximal to the PxXP motif harbors the central residues of the RT loop, the distal site covers a newly identified N-terminal helix of Nef (residues 60 to 69) with W61 mediating multiple interactions to hydrophobic residues L104, L113 and L114. The 8-fold increase in affinity of the Hck-SH3 RT-loop mutant VSWSPD compared to the EAIHHE wild type sequence is largely due to multiple interactions mediated by the central tryptophane residue. Importantly, this Trp residue positions the mutant R96I in human Fyn kinase, which was found to be a prerequisite and key site for the interaction between the SH3<sub>52</sub> domain and NLA-3 Nef [27,36]. The CD4 fragment of NI3-13 is unfortunately not resolved in the low resolution crystal structure of its complex with Nef. However, NMR spectroscopic analysis strongly support the displacement of the before mentioned newly identified N-terminal helix by the CD4 segment. Mutagenesis analyses showed that the CD4 dileucine motif is required for binding to Nef, while a C-terminal cysteine-rich motif, which was shown to form an intermolecular zinc-finger with the unique domain of Hck [33], did not contribute to the interaction. This suggested that the C-terminal residues of the CD4 segment did not contribute to the binary interaction significantly and its truncation to a fusion protein that encompassed 86 residues only (NI3-13) showed still a binding affinity of 41 nM. Together, these interaction data prove indeed the two hydrophobic cavities of Nef as potent pharmacophore target sites.

The design of protein inhibitors is often based on substrate modifications (e.g., histone deacetylase inhibitors, farnesyl transferase inhibitors) or modifications of a co-enzyme/co-substrate (e.g., ATP analogues for kinases) that block an enzymatic reaction. As such, inhibitors against reverse transcriptase, protease and integrase that constitute current HAART treatment target the active center of these enzymes. Combinations of these drugs are known to be highly effective, however, escape mutants that develop with time require constant monitoring and adjustment of the drug regime [2]. Other classes of inhibitors are fusion and attachment inhibitors against gp41 or CCR5 as well as maturation inhibitors targeting Gag that both impair structural factors of HIV. Fusion inhibitors have been designed in various fashions, ranging from 20 to 44 amino acid long D-peptides which interact with the tripartite coiled colloid of the glycoprotein, blocking fusion and thus entry of the virus to the target cell [47,48]. Finally, lead inhibitory molecules that target the HIV-1 capsid and prevent assembly of infectious virus particles have been identified and are currently being developed towards therapeutic application by a design approach to achieve cell penetration [49–52]. While current anti-HIV infectives target enzymes or structural proteins essential to viral replication, Nef might be regarded as an accessory adaptor protein that fulfills many functions by an assembly of multiple sequence motifs. The structural and functional insight gained by the “wrapping Nef” approach described here provide valuable lead information and proof-of-
concept that will guide future efforts targeted at the generation of potent and clinically applicable Nef inhibitors. Future work will match with the design, functional characterization and structure determination on Nef protein inhibitors provided in this study.

Materials and Methods

Cloning, expression and purification of Nef inhibitor constructs

HIV-1 Nef(1-44) proteins (GenBank accession number K02007) were expressed in E. coli BL21(DE3) cells from a codon optimized plasmid with a TEV protease cleavable N-terminal His10-tag using pProEx HTa (Stratagene) as expression vector or pET-23d (Novagen) with a C-terminal His6-tag. The expression and purification of wild type Nef or N-terminally truncated Nef (Δ1-27), Nef (Δ1-44), Nef (Δ1-58), and mutant Nef (P76A, P79A, R81A) all in combination with a C-terminal cysteine to alanine mutation (C210A) was performed similarly as described [53]. Briefly, bacterial cells were cultured in LB medium containing 100 μg/ml ampicillin at 30°C and induced at OD600 = 0.8 with 0.5 mM IPTG for additional 5 hrs. For NMR experiments Nef (Δ1-44) has been cultured in presence of minimal medium containing 1 g/l 15NH4Cl. The harvested cells were resuspended in 20 mM Tris/HCl (pH 8.0), 500 mM NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF, lyzed by fluidizer and cleared by spinning for 45 min at 30,000 g. The fusion proteins were separated from the bacterial proteins by affinity chromatography using Ni-NTA resin (Qiagen) and size exclusion chromatography on a Superdex 75 column (Amersham Bioscience), analyzed by SDS Page and MALDI-TOF mass spectrometry and stored in 10 mM Tris/HCl (pH 8.0), 50 mM NaCl at −80°C.

Fusion constructs of human Hck-SH3 domains (AC: M16391) with either the adaptor protein β2 subunit (M31475), the cytoplasmic tail of human CD4 (M12807) or the VHS domain of GGA2 (AF233522), were generated by PCR using a cassette system of restrictions sites with NdeI and EcoRI at the 5′ and 3′ end, respectively, and BamHI or Spel in between the domains. Typically, a glycine rich amino acid linker as GGGS or GGGSGGGS was cloned in between the domains. For in vitro experiments, proteins were expressed with an N-terminal glutathione S-transferase (GST)-tag using the pGEX-4T1 Tev vector system (Amersham Bioscience) in LB medium containing 100 μg/ml ampicillin at 30°C and induced at an OD600 = 0.8 with 0.5 mM IPTG for additional 8 h. The harvested cells were resuspended in 20 mM Tris/HCl (pH 8.0), 500 mM NaCl, 2 mM GSH, 1 mM EDTA, 0.5 mM DTE and 1 mM PMSF, lyzed by fluidizer and cleared by spinning for 45 min at 30,000 g. By affinity chromatography using GSH resin (Amersham Bioscience) and size exclusion chromatography on a Superdex 75 column (Amersham Bioscience) the fusion proteins were separated from the bacterial proteins, analyzed by SDS Page and MALDI-TOF mass spectrometry and stored in 10 mM Tris/HCl, pH 8.0, 50 mM NaCl at −80°C.

For cellular expression assays, Nef interacting proteins were optionally targeted to lipid membranes by an N-terminal myristoylation motif (S3C), since myristoylation alone was often shown to be not sufficient for stable membrane association [54,55]. Alternatively, a C-terminal palmitoylation and farnesylation motif (S3C), since myristoylation alone was often shown to be not sufficient for stable membrane association [34,54].

Complex crystallization and structure determination

For crystallization 15 mg ml−1 of purified Nef-NI1-2 protein complex was stored in 30 mM Hepes buffer, pH 8.0, and 50 mM NaCl. Crystallization drops were set at a 1:1 ratio with the reservoir solution containing 10% (w/v) PEG 8000, 0.2 M MgCl2, 15 mM MnCl2, 5% (v/v) ethylene glycol, 100 mM Tris buffer, pH 7.0, and subjected to hanging drop vapor diffusion at 12°C. Crystals appeared within three days and grew to a size of approx. 100 * 100 * 50 μm. They were briefly washed in mother liquor supplemented with 10% sucrose, 10% xylitol and 15% ethylene glycol prior to flash-cooling in liquid nitrogen. Nef-NI3-13 was crystallized in a similar fashion, using a reservoir of 0.1 M Hepes pH 7.0, 0.05 M sodium citrate, 15% isopropanol and 30% glycerol as a cryoprotectant.

Diffraction data were collected on beamline X10SA of the Swiss Light Source (SLS, Villigen, Switzerland) equipped with a MAR 225 CCD detector. The XDS package [56] was used for data reduction and the structure of the Nef–NI1-2 complex was solved by molecular replacement using the Nef-FynSH3 complex (PDB entry 1EFN [36]) as a search model in Phaser [57]. The model was refined to 2.0 Å resolution by alternating cycles of manual rebuilding in COOT [58] and minimization in REFMAC5 [59], defining each of the four chains contained in the asymmetric unit as a separate TLS body. The Nef–NI1-2 complex was solved by molecular replacement with the refined structure of Nef–NI1-2 and the structure was refined against 3.45 Å resolution data using COOT and phenix.refine [60]. Due to the low resolution, individual atomic displacement factor refinement was not
performed and B-factors were only derived from TLS refinement, refining the four chains as separate TLS groups. Data collection and refinement statistics are summarized in Table 2. Molecular diagrams were drawn using PyMOL (http://pymol.sourceforge.net/).

Nuclear magnetic resonance spectroscopy
NMR spectroscopy experiments were carried out with a Varian Inova 600 MHz spectrometer at 25 °C in 5 mM Tris/HCl (pH 8.0) buffer and 8% D2O. The 2D 1H/15N HSQC spectra were obtained by titrating 0.4–0.6 mM Nef with either N1-2 or the interacting fusion protein N13-13 to a final concentration of 0.8 mM. Spectra were analyzed based on the resonance line assignments of Nef, alleles BH10 and SF2 [61–63] using the program AURELIA [64].

Cells and Reagents
293T and TZM cells were purchased from ATCC (Teddington, UK), 293T, TZM (JCl3BL), and TZM clone 13 cells (subcloned for high expression of MHC-I by fluorescence-activated cell sorting) were maintained in DMEM high medium ( Gibco) supplemented with 10% fetal calf serum ( Invitrogen), L-glutamine and antibiotics (both from Gibco) as described [5]. CHO hCD4hCCR5 (stably expressing hCD4 and hCCR5) [65] cells were cultured in RPMI 1640 medium supplemented accordingly.

Mammalian expression plasmids
The expression plasmid encoding wild-type HIV-1 SF2 Nef as a GFP fusion protein (from the pEGFP-N1 vector; Clontech) has been described previously [65]. The proviral construct expressing GFP fusion protein (from the pEGFP-N1 vector; Clontech) has assignments of Nef, alleles BH10 and SF2 [61–63] using the program AURELIA [64].

Immunofluorescence
To visualize the subcellular localization of Nef, GFP and HA-tagged inhibitors, CHO hCD4hCCR5 cells were seeded onto glass coverslips and transfected with corresponding expression plasmids. 36 hours later, cells were fixed with 4% paraformaldehyde/ phosphate-buffered saline (10 min at room temperature), permeabilized with 0.1% Tx-100, blocked for 1 h with 1% BSA in PBS, and stained with HA-antibody (F7; Santa Cruz Biotechnology) followed by appropriate fluorescent secondary antibody (Alexa568, Molecular Probes). Following extensive washing, cells were mounted with HistoPrep (Linaris). Confocal microscopy images were acquired with a LSM 510 confocal laser scanning microscope (Zeiss). Final images were processed in Photoshop CS2 (Adobe Systems).

Western Blotting
For Western blot analysis, post-nuclear supernatants were boiled in SDS sample buffer, separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Protein detection was performed following incubation with appropriate first and secondary antibodies using the super signal pico detection kit (Pierce) according to the manufacturer’s instructions.

Co-Immunoprecipitation
CHO hCD4hCCR5 cells were lysed in IP-buffser [25 mM Tris/HCl pH 7.4; 150 mM NaCl, 1 mM EDTA, 1% NP-40] containing protease inhibitors. After removal of nuclei and cell debris by centrifugation at 1,000 g for 10 min at 4 °C, lysates were incubated over night at 4 °C with prepared GFP-protein A-Sepharose beads (anti-GFP-antibodies incubated with protein A-Sepharose (Pharmacia) for 2 h at room temperature in IP-buffer 1 (25 mM Tris/HCl pH 7.4; 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) and washed in IP-buffer 2 (25 mM Tris/HCl pH 7.4; 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% BSA). Samples were pelleted, washed with IP-buffer 0 (25 mM Tris/HCl pH 7.4; 150 mM NaCl, 1 mM EDTA), and resuspended in sample-buffer. After SDS-PAGE, western blotting with anti-HA-mAb was performed.

Flow cytometry
24 h post-transfection surface molecules of Chinese hamster ovary (CHO) hCD4hCCR5 or TZM clone 13 cells were stained, respectively as reported previously [22,65]. Staining was performed at 4 °C for 30 min in fluorescence-activated cell sorting (FACS) medium (3% FCS, 0.05% sodium azide in PBS) with anti-hCD4-APC, anti-hCCR5-APC (both BD Pharmingen), anti-CD71 (H68.6; Zymed Laboratories), and anti-MHC-I (W6/32-FTIC, Sigma-Aldrich) antibodies, respectively. For untagged anti-CD71 mAb, secondary staining was performed with APC conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Samples were analyzed by flow cytometry using a FACSCalibur cytometer (Becton Dickinson).

Supporting Information
Figure S1 Schematic display of the four generations of inhibitor constructs used in cellular experiments. Domain boundaries, membrane targeting sites and sequence epitopes attached for antibody recognition in vivo are indicated. Domain boundaries for the human Hck SH3 domain are given according to the UniProt database entry P08631-1, described as isoform 1 of protein product p60-Hck. Residue numbering of human CD4 is assigned according to UniProt protein entry P01730. All protein products besides N13-6 were targeted to cellular membranes either by an N-terminal myristoylation motif or a C-terminal farnesylation signal, partly in combination with an additional palmitoylation signal for increased membrane association, which is schematically indicated as carbon structure (zigzag lines) (TIF).

Figure S2 Fluorescence spectroscopy analysis of the binding interaction between Nef and N13-3. (A) Emission spectra of fluorescence-labeled N13-3 Hexim1 alone and in complex with Nef (1–210) indicate complex formation by an increase and shift in fluorescence emission. (B) Analysis of an
equilibrium titration series of N13-3-EDANS with increasing concentrations of Nef (1-210) indicated a dissociation constant $K_d$ of 37 nM. The relative fluorescence intensity was corrected for the dilution effects.

Figure S3 Surface plasmon resonance measurements of Nef binding to an inhibitor of the third generation. (A) Nef3F2 (45-210) was floated at four different concentrations (0.4 to 1.0 μM) over GST-N13-9 which was immobilized on the chip surface of the SPR biosensor. Following the association step Nef protein was washed off after 400 s by Guanidine hydrochloride. (B) The concentration dependent analysis of the association and dissociation reaction revealed a dissociation constant of 28 nM for the Nef-GST-N13-9 interaction.

Figure S4 Size exclusion chromatography of Nef and the Nef-N13-3 protein complex. While Nef (45-210) elutes at its expected size of ~28 kDa as similarly observed before [53], the Nef-N13-3 complex elutes at a molecular weight of approximately 38 kDa. These observations suggest a heterodimeric but not a heterotetrameric complex assembly, indicating that the gain in affinity by the CD4 fraction in N13-3 is achieved by the interaction with the same Nef molecule. Note that the small portion of oligomerized Nef in the void volume at 8.2 ml disappeared upon addition of Nef inhibitor.

Figure S5 Expression and localization of NI3-1 to NI3-12 in human cells. (A) Localization of Nef and Nb in HeLa cells. GFP or NefGFP was co-expressed with an empty control vector in human cells. (TIF)

Nef binding to an inhibitor of the third generation. (A) Nef3F2 (45-210) was floated at four different concentrations (0.4 to 1.0 μM) over GST-N13-9 which was immobilized on the chip surface of the SPR biosensor. Following the association step Nef protein was washed off after 400 s by Guanidine hydrochloride. (B) The concentration dependent analysis of the association and dissociation reaction revealed a dissociation constant of 28 nM for the Nef-GST-N13-9 interaction.

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Western blot analysis of lysates of the cells shown in (A) using an anti-HA antibody for detection of the indicated NI proteins.

Figure S6 Structural details of the complex interface between Nef3F2 and N13-2. (A) The central residues Y89VSWSPDD of the mutated RT loop tightly interact with helices α2 and α5 of Nef. Particularly the indol ring of W92N11-2 performs multiple interactions with hydrophobic residues I91, F94, W117 and I118 of Nef. (B) A polar cluster between S93 and D95 of N11-2 and K86 and D90 of Nef sustains the complex binding. (C) Interactions of the newly identified N-terminal helix (60–69) of Nef with its core domain structure. Tryptophane 61 undergoes tight interactions with L104, R110, I113 and L114 of the Nef core domain structure, covering the distal hydrophobic crevice of the α2 and α5 helices.

Figure S7 NMR titration series of Nef3F2 (45-210, C59S, C210S) with inhibitor construct N13-13. Shown are 15N/1H HSQC spectra of 15N labeled Nef in the initial, unperturbed state (blue resonance signals), followed by addition of N13-13 at a molar ratio to Nef of 0.5 (orange lines) and 1.2 (red line).

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Author Contributions

Conceived and designed the experiments: SB OTF MG. Performed the experiments: SB SIS AS WB. Analyzed the data: SB SIS AS WB OTF MG. Wrote the paper: OTF MG.

References

1. Gupta RK, Hill A, Sawyer AW, Cozzi-Lepri A, von Wyl V, et al. (2009) Virological monitoring and resistance to first-line highly active antiretroviral therapy in adults infected with HIV-1 treated under WHO guidelines: a systematic review and meta-analysis. Lancet Infect Dis 9: 609–417.
2. Flexner C (2007) HIV drug development: the next 25 years. Nat Rev Drug Discov 6: 351: 322–339.
3. Kestler HW, 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, et al. (1991) HIV-1 Nef. EMBO Rep 2: 580–583.
4. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, et al. (1995) The Nef interferes with host cell motility by deregulation of Cofilin. Cell Host 125: 1055–1067.
5. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC (1995) Importance of the nef gene for maintenance of high virus loads and for major determinant of pathogenicity for an AIDS-like disease induced by HIV-1. Cell 125: 1055–1067.
6. Jere A, Fujita M, Adachi A, Nomaguchi M (2010) Role of HIV-1 Nef protein for virus replication in vitro. Microbes Infect 12: 63–70.
7. Haller C, Tibroni N, Rudolph JM, Grosse R, Fackler OT (2011) Nef does not inhibit F-actin remodelling and HIV-1 cell-cell transmission at the T lymphocyte virological synapse. Eur J Cell Biol, in press, doi:10.1016/j.ejcb.2010.09.010.
8. Betz S, Restoniu A, Opi S, Arolf ST, Parrot I, et al. (2007) Protein protein interaction inhibition (2P2I) combining high throughput and virtual screening: Application to the HIV-1 Nef protein. Proc Natl Acad Sci U S A 104: 19256–19261.
9. Olszewski A, Sato K, Aron ZD, Cohen F, Harris A, et al. (2004) Guanidine alkaloid analogs as inhibitors of HIV-1 Nef interactions with p53, actin, and p56lck. Proc Natl Acad Sci U S A 101: 14079–14084.
10. Emerit-Sellak I, Kodama T, Lerney EC, Dai W, Foster C, et al. (2009) Chemical library screens targeting an HIV-1 accessory factor/host cell kinase complex identify novel antiretroviral compounds. ACS Chem Biol 4: 939–947.
11. Delekos JD, Atkins KM, Thomas L, Emerit-Sellak I, Byeon IJ, et al. (2010) Small molecule inhibition of HIV-1-induced MHC-I down-regulation identifies a temporally regulated switch in Nef action. Mol Biol Cell 21: 3279–3292.
12. Geyer M, Peterlin BM (2001) Domain assembly, surface accessibility and sequence conservation in full length HIV-1 Nef. FEBS Lett 496: 91–95.
13. Olszewski A, Piquet V, Wang JK, Chen YL, Tatro D (1999) Nef-induced CD4 and major histocompatibility complex class I MHC-I down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. J Virol 73: 1964–1973.
14. Michel N, Allespach I, Venzke S, Fackler OT, Keppler OT (2005) The Nef protein of human immunodeficiency virus establishes superinfection immunity by a dual strategy to downregulate cell-surface CCR5 and CD4. Curr Biol 15: 714–723.
15. Hirakawa M, Hsuata P, Manninen A, Renkema GH, Sakela K (2001) Inhibition of cellular functions of HIV-1 Nef by artificial HIV-1 nef constructs. Virology 286: 152–159.
16. Craig HM, Pandori MW, Guatelli JC (1998) Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. Proc Natl Acad Sci U S A 95: 11229–11234.
17. Geyer M, Yu H, Mandle R, Linnenmann T, Zheng YH, et al. (2002) Subunit H of the V-ATPase binds to the medium chain of adaptin protein complex 2 and connects Nef to the endocytotic machinery. J Biol Chem 277: 28521–28529.
26. Grzesiek S, Stahl SJ, Wingfield PT, Bab A (1996) The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. Biochemistry 35: 10256–10261.
27. Lee CH, Leung B, Lennson MA, Zheng J, Cowburn D, et al. (1995) A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. EMBO J 14: 5006–5015.
28. H Epstein K, Poitouen K, Sakaela K (1999) SH3 domains with high affinity and engineered ligand specificities targeted to HIV-1 Nef. J Mol Biol 293: 1097–1106.
29. Geyer M, Fackler OT, Peterlin BM (2002) Subunit H of the V-ATPase involved in endolysosome shows homology to beta-adaptins. Mol Biol Cell 13: 2045–2056.
30. Mura S, Purtollano R, Kato Y, Bonilha JS, Hurley JH (2002) Structural basis for acidic-cluster-dileucine sorting-signal recognition by VHS domains. Nat. 415: 933–937.
31. Shiha T, Takatsu H, Nogoi T, Matsuumi N, Kawasumi M, et al. (2002) Structural basis for recognition of acidic-cluster dileucine sequence by GGA1. Nature 415: 937–941.
32. Arolt S, O'Brien R, Franken P, Strub MP, Hoh F, et al. (1998) RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef. Biochemistry 37: 14683–14691.
33. Piqueu V, Sun YZ, Blacklow SC, Wagner G, Eck MJ (2003) A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. Science 301: 1725–1728.
34. Gerlach H, Laumann V, Martens S, Becker CF, Goody RS, et al. (2010) HIV-1 Nef membrane association depends on charge, curvature, composition and sequence. Nat Chem 6: 46–53.
35. Aiken C, Trono D (1995) Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J Virol 69: 5049–5056.
36. Lee CH, Sakela K, Mira UA, Chai BT, Kurjan J (1996) Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell 85: 931–942.
37. Arolt S, Franken P, Strub MP, Hoh F, Bengsch E, et al. (1997) The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling. Structure 5: 1361–1372.
38. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW (1996) Discovering high-affinity ligands for proteins: SAR by NMR. Science 274: 1531–1534.
39. Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov 3: 301–317.
40. Piguet V, Sun ZY, Blacklow SC, Wagner G, Eck MJ (2000) HIV-1 Nef protein binds to the cellular protein PACS-1 to downregulate class I major histocompatibility complex. Nat Cell Biol 2: 163–167.
41. Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov 3: 301–317.