Published in final edited form as:
J Neuroimmune Pharmacol. 2011 June ; 6(2): 230–246. doi:10.1007/s11481-011-9262-y.

Mechanisms of HIV-1 Nef Function and Intracellular Signaling

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Abstract

Advances in the last several years have enhanced mechanistic understanding of Nef induced CD4 and MHCI downregulation and have suggested a new paradigm for analyzing Nef function. In both of these cases, Nef acts by forming ternary complexes with significant contributions to stability imparted by non-canonical interactions. The mutational analyses and binding assays that have led to these conclusions are discussed. The recent progress has been dependent on conservative mutations and multi-protein binding assays. The poorly understood Nef functions of p21 activated protein kinase (PAK2) activation, enhancement of virion infectivity, and inhibition of immunoglobulin class switching are also likely to involve ternary complexes and non-canonical interactions. Hence, investigation of these latter Nef functions should benefit from a similar approach.

Six historically used alanine substitutions for determining structure-function relationships of Nef are discussed. These are M20A, E62A/E63A/E64A/E65A (AAAA), P72A/P75A (AXXA), R106A, L164A/L165A, and D174A/D175A. Investigations of less disruptive mutations in place of AAAA and AXXA have led to different interpretations of mechanism. Two recent examples of this alternate approach applied to PAK2 activation F191 and critical residue D123 are presented.

The implications of the new findings and the resulting new paradigm for Nef structure-function are discussed with respect to creating a map of Nef functions on the protein surface. We report the results of a PPI-Pred analysis for protein-protein interfaces. There are three predicted patches produced by the analysis which describe regions consistent with the currently known mutational analyses of Nef function.

Keywords

HIV-1; Nef; non-canonical; CD4; MHCI; PAK2; infectivity; immunoglobulin class switching

Introduction

The human immunodeficiency virus type I (HIV-1) accessory gene product, Nef, is a myristoylated protein with a decisive role in viral replication and pathogenesis (Kestler et al., 1991; Foster and Garcia, 2007; Gorry et al., 2007; Kirchhoff et al., 2008). HIV-1Nef is only 206 amino acids, but is functionally complex. Structurally, this complexity is reflected

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The authors declare that they have no competing interests.

in overlapping effector domains that interact with multiple cellular proteins. These interactions bring about abnormal associations of host cell proteins that establish a favorable environment for viral replication (Arold et al., 1997; O'Neill et al., 2006b; Kirchhoff et al., 2008; Lindwasser et al., 2008; Noviello et al., 2008). HIV-1Nef has a structured core: amino acids 62–147 and 179–200; flexible N-and C- termini: amino acids 2–61 and 201–206; and an internal flexible loop: amino acids 148–178 (standard NL4–3 numbering).

Nef is considered to be a pathogenic factor, but its role as an inducer of AIDS is not mechanistically understood. One possibility is that Nef acts overall to maintain high levels of HIV-1 replication as a result of a synergism between its many activities (Fackler et al., 2007). Alternately, a single Nef activity may largely account for the protein's pathogenic impact. Evidence from the SIV_{MAC239}/rhesus macaque model of pathogenesis would suggest CD4 downregulation as the critical factor with MHCI downregulation and PAK2 activation playing lesser roles (Lang et al., 1997; Carl et al., 2000; Iafrate et al., 2000; Swigut et al., 2004).

Myristoylation is critical to all Nef functions with only one exception, the activation of the tyrosine kinase, Hck (Briggs et al., 2001). Myristoylation-dependent Nef functions include redirection of the transiting of host cell membrane proteins, particularly the downregulation of cell surface CD4 and MHCI. Nef also requires myristoylation to activate cell signaling pathways, with enhancement of p21-activated protein kinase (PAK2) autophosphorylation having received the most attention. Additional examples are the enhancement of HIV-1 virion infectivity and inhibition of immunoglobulin class switching. These last two activities are extremely complex and poorly understood. In the case of the enhancement of virion infectivity Nef acts within the infected cell to alter virions prior to release in such a manner that subsequent infection of a new cell is more efficient (Goldsmith et al., 1995; Madrid et al., 2005; Pizzato et al., 2007). The attenuation of immunoglobulin class switching by Nef also involves two cells. Infected macrophages transport Nef through long range B cell-targeting conduits. Nef not only induces the formation of the conduits, but also acts as the signal within the B cell to suppress IgG2 and IgA production (Qiao et al., 2006; Xu et al., 2009). While Nef acts at cellular membranes to downregulate CD4 and MHCI, activate PAK2, enhance virion infectivity, and inhibit immunoglobulin class switching, it does so at diverse cellular locations. Myristoylation allows Nef to act at these different membrane compartments by the simple mechanism of dissociation/reassociation from the membrane (Kwak et al.; Yi et al.).

Although Nef effects all of its activities by protein-protein interactions, only one host cell protein, the protein tyrosine kinase Hck, has been found to bind directly with high affinity to HIV-1 Nef (K_D approximately 200 nM) (Lee et al., 1996; Karkkainen et al., 2006). The binding of Nef activates Hck (Saksela et al., 1995; Briggs et al., 1997; Picard et al., 2002; Kim et al., 2006) which is present in monocytic cells, including microglia, but not T cells (Krady et al., 2002). The limited tissue distribution of Hck is expected to limit the pathogenic potential for Hck activation and this result has been observed by Jolicoeur and co-workers. In their transgenic mouse model, the expression of Nef from a human CD4 promoter and mouse CD4 enhancer combination ($CD4C/HIV^{Nef}$), results in a multi-organ, lethal disease with symptoms similar to pediatric AIDS (Hanna et al., 1998b; Hanna et al., 1998a). Expressing Nef from the transgene in a $hck^{-/-}$ mouse did not prevent pathogenic symptoms, but prolonged the latency of disease development (Hanna et al., 2001). From these results Hanna et al. suggested that Hck has a role in Nef induced organ disease, but that other factors may be more critical.

Recent evidence suggests that Nef may act in the $CD4C/HIV^{Nef}$ transgenic mouse model during development in cells not infected in HIV-1 disease. To address this question an

advanced transgenic mouse model was developed using the tet-On system to suppress Nef expression. Adding doxycycline to the drinking water of adult mice induced Nef expression (Kistner et al., 1996; Rahim et al., 2009). Despite the very low level of Nef expression in the absence of inducer, doxycyclin fully induced Nef expression in adult thymus, but not in double negative thymocytes. These mice did not develop organ disease, but exhibited significant reductions in CD4+, CD8+ and CD4+CD8+ T cells in thymus. Other thymic effects were generation of $CD4^{low}$ T cell subsets, elevated levels of phosphotyrosine, and impaired maturation of single positive thymocytes. In peripheral lymphoid tissues induction of Nef led to downregulation of CD4 surface expression, an increased proportion of apoptotic CD4+ T cells, and depletion of T lymphocytes. Whether there is a role for Hck in these doxycyclin-induced effects in adult mice is unknown. With this elegant model, effects can be studied that result from Nef's downregulation of mouse CD4, activation of mouse PAK2, and enhancement of T cell activation, but not enhancement of infectivity or MHCI downregulation (Rhee and Marsh, 1994; Sawai et al., 1995; Fleis et al., 2002). It would be potentially very interesting if Nef induced inhibition of class switching in this system.

For all Nef phenotypes other than Hck activation, the cellular proteins involved are not fully defined, but it is very likely that in each case ternary or higher complexes of cellular proteins bound to Nef are responsible. Strong evidence indicates that to downregulate CD4, Nef forms a ternary complex with adapter protein 2 (AP2) and the cytoplasmic tail of CD4 (Lindwasser et al., 2008; Chaudhuri et al., 2009). For MHCI downregulation, Nef binds to adapter protein 1 (AP1) and the cytoplasmic tail of MHCI. Nef also binds to PAK2 in a complex with other cellular proteins probably including a GTPase and a GEF (Rauch et al., 2008). With the exception of dynamin 2 the cellular proteins that interact with Nef to enhance virion infectivity are unknown but mutational analysis of Nef suggests that both protein trafficking and cell signaling derangements are involved (Madrid et al., 2005; Pizzato et al., 2007). Inhibition of class switching is even more complex involving structural alterations in the macrophage cells expressing Nef and subsequent signaling in B cells (Xu et al., 2009).

CD4 downregulation

The mechanism of CD4 downregulation is the best understood of all Nef activities. Nef binds the cytoplasmic tail of CD4 with a K_D of about 1μM. Nef amino acids 57–59, 95, 97, 106, 110 and additional residues in the N-terminal flexible arm of Nef form the interface with CD4 (Grzesiek et al., 1996a; Preusser et al., 2001). This binding is not thought to be sufficient for Nef to downregulate CD4 from the plasma membrane (Preusser et al., 2001). AP-2 associates with Nef to bring about internalization of CD4 (Chaudhuri et al., 2007; Lindwasser et al., 2008). It is important to note that the association of Nef with AP-2 requires not only the adjacent leucines within the dileucine motif, ENNSLL (amino acids 160–165), but also a second region known as the diacidic motif, (D/E)E (amino acids 174 and 175) (Lindwasser et al., 2008; Chaudhuri et al., 2009). Demonstration of Nef, AP-2, and the cytoplasmic tail of CD4 in a tripartite complex required a Y4H system. In this system, Nef and CD4 failed to interact in the absence of the co-expression of both AP-2 α and σ2 proteins (Chaudhuri et al., 2009). As indicated by daSilva et al., simply accelerating the internalization of CD4 would not significantly decrease the level of the protein on the plasma membrane unless recycling back to the cell surface is also suppressed (daSilva et al., 2009). Nef also induces targeting of CD4 to lysosomes (Luo et al., 1996; daSilva et al., 2009). As a result, the total amount of CD4 in Nef expressing cells is reduced (Anderson et al., 1994; daSilva et al., 2009). The current leading candidate as the cellular target for Nef in this case is ESCRT (Costa et al., 2006; daSilva et al., 2009). Initial evidence suggests that this process is independent of ubiquitination (daSilva et al., 2009).

In uninfected T cells, the CD4 cytoplasmic tail is tightly bound to Lck, and this complex is refractory to internalization from the cell membrane. Following infection, Nef is able to disrupt CD4-Lck association by an unknown mechanism, and internalize Lck to endosomes. However, internalization of CD4 and the targeting of CD4 and Lck to lysosomes are genetically separable processes (Thoulouze et al., 2006; Haller et al., 2007; Laguette et al., 2009b).

MHCI downregulation

MHCI downregulation is not as well understood as CD4 downregulation, but presents some interesting parallels. In this case a ternary complex has been proposed between Nef, AP-1, and the cytoplasmic tail of MHCI. Nef induces a ternary complex which has a non-canonical interaction between the cytoplasmic tail of MHCI with the YXXφ binding site of AP-1 (Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). YXXφ (φ is an amino acid with a bulky hydrophobic side chain) is a tyrosine based storing signal (Bonifacino and Traub, 2003). The suggested role of Nef is to compensate for the presence of an alanine instead of φ in the MHCI cytoplasmic tail (YSQA) (Wonderlich et al., 2008). This ternary complex can only be demonstrated in vitro when the MHCI cytoplasmic tail is fused to the N-terminus of Nef that is mutated to inactivate the dileucine motif by mutating leucines 164 and 165 to alanines (MHCIct-NefLLAA) (Coleman et al., 2006; Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). The dileucine mutation is necessary because native Nef also binds directly to AP-1 in vitro at the canonical (D/E)XXXL(L/I) binding site (Bonifacino and Traub, 2003). Mutation of a run of four glutamates (EEEE, amino acids 62–65) will also prevent the in vitro binding of AP-1 to the MHCIct-NefLLAA (Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). In addition, mutation of M20 or P78 to alanine prevents MHCI downregulation, but the roles of these residues in the ternary complex are not yet defined (Akari et al., 2000; Yamada et al., 2003; Casartelli et al., 2006; Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). This model of Nef downregulation of MHCI predicts that the ternary complex interacts with additional cellular factors to direct newly synthesized MHCI to lysosomes for degradation instead of the default pathway leading to the cell surface (Noviello et al., 2008; Schaefer et al., 2008; Wonderlich et al., 2008; Singh et al., 2009).

An alternate model of Thomas and co-workers invokes a much more complicated model. In the original version the tetraglutamate segment of Nef, EEEE, was viewed as mimicking the acidic cluster in the cytoplasmic tail of furin to bind PACS-1 (Piguet et al., 1999; Blagoveshchenskaya et al., 2002). The furin acidic cluster is SDSEEDE and is only active in binding PACS-1 when the serines are phosphorylated. The double mutant DDDEEDE is a constitutively active phosophomimic and the double mutant ADAEEDE is a constitutively inactive dephosphomimic. The interpretation that EEEE in Nef acted as an acidic cluster was questioned by Baugh et al when it was noted that substitution of the ADAEEDE sequence into Nef did not prevent MHCI downregulation. The in vitro binding of Nef with the furin binding domain of PACS-1 was noted to be very weak relative to the binding of the cytoplasmic tail of furin to PACS-1 (Baugh et al., 2008). Subsequently, the part of the furin acidic cluster that is similar to Nef (EEDE) has been assigned a separate function while the phosphoserines are considered to be required for PACS-1 binding by furin. (Simmen et al., 1999; Atkins et al., 2008; Youker et al., 2009). In the model presented in Blagoveshchenskaya et al. the binding of Nef to PACS-1 led to a chain of events resulting in the activation of PI3K. Subsequent elaboration of the model has replaced PACS-1 with PACS-2, described a complex of four proteins including Nef, PI3K, ZAP70/Syk and one of three Src family kinases, Hck, Lyn, or Src (Dikeakos et al.; Hung et al., 2007; Atkins et al., 2008). This pathway has been labeled as the signaling mechanism for MHCI downregulation since it is proposed that it brings about elevated PIP_3 that activates ARF6 to accelerate

MHCI endocytosis from the cell surface (Youker et al., 2009). It is not clear that the signaling pathway is able to lower the amount of MHCI on the cell surface since a downstream process dependent on the M20 residue is required to remove MHCI from the cell surface. The mutant NefM20A results in increased MHCI recycling at the cell surface without reducing the level of MHCI at the cell surface (Blagoveshchenskaya et al., 2002). Thus, the model of Thomas and co-workers includes an M20-dependent Nef activity that retains MHCI within the cell interior. This activity may be the same process as that described by the models of Collins and co-workers and Guatelli and co-workers (Youker et al., 2009).

Neither of the presented models have a role for Nef binding to the cytoplasmic tail of MHCI at the plasma membrane, but Yi, et al. have observed Nef binding to HLA-1 at the endoplasmic reticulum, Golgi/trans-Golgi network, and the plasma membrane (Yi et al.; Noviello et al., 2008; Wonderlich et al., 2008; Youker et al., 2009). While the mechanism of downregulation of MHCI remains to be elucidated it is clear that this Nef function can prevent cytolysis of infected cells by cytotoxic T cells in vitro (Collins et al., 1998; Yang et al., 2002). However, a major unaddressed question in the Nef field is the inability of Nef-induced downregulation of MHCI to maintain viral replication in elite suppressors (Pereyra et al.; Bailey et al., 2008; Bailey et al., 2009).

New paradigm of Nef function

The advances in our understanding of how Nef manipulates CD4 and MHCI protein trafficking, suggest a general approach for the investigation of Nef function. First, Nef acts by forming ternary and possible higher complexes with cellular proteins. Despite its small size, this protein engages in interactions with multiple host cell proteins. In general, these complex interactions are not amenable to elucidation by pull down assays and other experimental approaches for detecting direct binding. To gain mechanistic understanding of Nef functions requires additional information to be able to perform targeted binding experiments that reflect the formation of ternary or higher complexes between Nef and cellular proteins (Chaudhuri et al., 2009; Singh et al., 2009). A second prominent feature of the mechanism by which Nef alters cellular pathways is utilization of non-canonical interactions. An example is the demonstration of AP-2 as Nef's interacting partner for the downregulation of CD4. The role of AP-2 was initially obscured by the particularly weak binding of Nef to AP-2 in vitro and the limited impact of AP-2 siRNA knockdowns on CD4 downregulation. (Bresnahan et al., 1999; Rose et al., 2005; Chaudhuri et al., 2009). Hence, it was important to know that PMA induced CD4 internalization occurs at the level of the plasma membrane suggesting AP-2 involvement (Pelchen-Matthews et al., 1998). A novel approach using Drosophila S2 cells confirmed this expectation (Chaudhuri et al., 2007). Also, alanine scanning mutagenesis was important for revealing the diacidic motif, (D/E)D, as a possible site of interaction between Nef and AP-2 (Aiken et al., 1996). Similarly for MHCI downregulation it was important to know that a likely site of Nef action was at the level of the TGN. This fact suggested AP-1 as a critical cellular factor (Roeth and Collins, 2006). These assumptions made it possible to design the appropriate tests, with Y4H for Nef-CD4 and MHCIct-NefLLAA for Nef-MHCI, to demonstrate ternary interactions (Chaudhuri et al., 2007; Noviello et al., 2008; Wonderlich et al., 2008; Chaudhuri et al., 2009). We consider these recent findings to strongly support a paradigm shift in the investigation of Nef functions (other than Hck binding) away from the assumption that Nef forms 1:1 complexes with host cell proteins by mimicking canonical interaction motifs. In general, Nef appears to utilize non-canonical interactions in ternary and possibly higher complexes. This perspective of Nef structure-function will be important in future investigations of poorly understood Nef activities such as PAK2 activation, enhancement of virion infectivity, and inhibition of class switching.

PAK2 activation

Formation of an activation complex between Nef and PAK2 has been refractory to efforts to gain a mechanistic understanding. Complex formation results in the activation of PAK2 autophosphorylation (Arora et al., 2000). The nature of the Nef/PAK2 complex is not understood, but is thought to include additional cellular proteins. It is clear that the complex is stabilized by high affinity hydrophobic interactions as it is resistant to 1M $MgCl_2$ (Foster et al., 2001). However, characterization of the components of the complex has been very difficult since it represents a small fraction of total cellular Nef and PAK2 (Sawai et al., 1995; Arora et al., 2000; Pulkkinen et al., 2004). Although the K_D of the Nef-PAK2 interaction remains unknown, the stability of the association and its conservation in simian and human lentiviruses is suggestive of a biological role (Van den Broeke et al.; Kirchhoff et al., 2004).

Evidence has been presented suggesting three potential effector loci on Nef for PAK2 activation. One interface is the SH3-binding domain, PQVPLR, spanning amino acids 72–77. Mutation of the two prolines to alanines, AQVALR, gives a multiply defective Nef protein. With this mutation, not only is PAK2 activation lost, but also MHCI downregulation, and the ability of Nef to enhance virion infectivity. CD4 downregulation is retained (Goldsmith et al., 1995; Greenberg et al., 1998; Blagoveshchenskaya et al., 2002; Rauch et al., 2008). Two reports suggest that the PQVPLR interface may interact differently for the unknown SH3 domain protein needed for PAK2 activation than for its binding to Hck. The mutant, NefP69A, is strongly reduced in its ability to activate PAK2, but not for Hck binding (Wiskerchen and Cheng-Mayer, 1996; Manninen et al., 1998).

A second possible site of protein-protein activation includes the highly conserved Nef residue R106 at the N-terminal end of α-helix 2 (Grzesiek et al., 1997) The R106A mutation severely reduces Nef-induced PAK2 activation, but like the AQVALR mutation, it is not specific for PAK2 activation. NefR106A is also defective for enhancement of infectivity and partially defective for CD4 and MHCI downregulation (Manninen et al., 1998; O'Neill et al., 2006b). R106 is one of eight Nef residues that have been suggested to serve as the core of a Nef dimer interface (Kwak et al.).

A third putative interface containing amino acids 85, 89, 187, 188, and 191 has been proposed (Agopian et al., 2006; O'Neill et al., 2006b; Agopian et al., 2007). Depending on the Nef allele, mutations of these Nef residues often alter the activation of PAK2. The five amino acids are close together on the surface of the Nef protein, but this binding surface appears to vary as to its exact location depending on HIV-1 subtype (O'Neill et al., 2006b). Subtype B Nefs have L at 85, H at 89, R at 188, and F at 191 (designated LHRF). Mutation of F191 to R, H, I, Y, L, or A results in loss of Nef induced PAK2 activation (O'Neill et al., 2006b; Rauch et al., 2008). Remarkably subtype E Nefs have F at 85, F at 89, A at 188 and R at 191(FFAR), and a substantial fraction of subtype C Nefs has F at 85, F at 89, H at 188, and H at 191 (FFHH). Despite the presence of R or H at Nef residue 191, these Nefs do activate PAK2 suggesting a compensatory mechanism for maintaining PAK2 activation in subtype E and subtype C Nefs (O'Neill et al., 2006a; O'Neill et al., 2006b). Therefore, this region of HIV-1 Nef appears to be unusual in that it has a recent history of structural variation while maintaining, competence to activate PAK2 (Agopian et al., 2006; O'Neill et al., 2006b). Unlike the mutations AQVALR and R106A, this third class of mutations is highly specific for PAK2 activation (O'Neill et al., 2006b; Schindler et al., 2007). We have proposed that Nefs with F191, R191, or H191 in the PAK2 activation interface may bind to different cellular proteins of unknown identity (O'Neill et al., 2006b). Alternatively, this putative protein interaction interface may be part of a Nef dimerization domain (Kwak et

al.). The discovery of a third PAK2 activation interface strongly indicates that the Nef-PAK2 activation complex contains three or more proteins.

Enhancement of virion infectivity and inhibition of class switching

Enhancement of virion infectivity can be demonstrated by a single round infection assay. Despite this infection occurring in indicator cells that are modified HeLa cells (Kimpton and Emerman, 1992), there is a strong requirement for Nef. Nef acts in the producer cell, not the target cell (Laguette et al., 2009a). The fact that virion infectivity can be greatly enhanced by both lysosome and proteosome inhibitors, suggests stabilization of the HIV-1 preintegration complex as the mechanism of the Nef effect (Wei et al., 2005). Nef mutated at either the dileucine motif (ENNSLL) or the SH3 binding region (PQVPLR) fails to enhance HIV-1 virion infectivity (Goldsmith et al., 1995; Madrid et al., 2005; Coleman et al., 2006). Thus, it appears that the enhancement of infectivity represents a complex function that requires mis-regulation of both protein trafficking and cell signaling pathways. The integration of trafficking and cell signaling pathways suggests another multi-protein complex between Nef and host cell proteins,

Inhibition of class switching involves the formation of long-range actin-propelled conduits in response to Nef. These conduits transport Nef from the infected macrophages to B cells in systemic and intestinal lymphoid follicles. This process is entirely dependent on myristoylation of Nef and the dileucine motif (L164/L165). Partial reductions in the process are observed for Nefs with P72A/P75A and E62A/E63A/E64A/E65A mutations (Xu et al., 2009).

Mutational analysis of Nef function

The two decades of Nef investigation have led to the development of a number of standard mutations that with varying degrees of specificity eliminate Nef function(s). These mutations have been employed after their initial descriptions by multiple laboratories. In light of the structural/functional complexity of Nef discussed above, we suggest that, in general, these mutations are not sufficiently specific to be the basis for mechanistic conclusions. The standard designations for these mutations are M20A (Akari et al., 2000), AAAA and AXXA (Greenberg et al., 1998), R106A and EDAA (Aiken et al., 1996), and LLAA (Iafrate et al., 1997). They have been used for investigations of CD4 downregulation, MHCI downregulation, PAK2 activation, enhancement of virion infectivity, and inhibition of class switching. The importance of these mutations in Nef research will be discussed along with the limitations inherent in the use of non-conservative mutations. Another commonly used mutation, G2A, prevents myristoylation. This mutation results in a general disruption of Nef function, leading to the important conclusion that Nef almost exclusively acts at cellular membranes. Finally, the mutations of phenylalanine-191 to isoleucine (F191I) that was introduced as a specific mutation of PAK2 activation and aspartate-123 to glutamate (D123E) for investigating multiple Nef phenotypes will be discussed (O'Neill et al., 2006b).

M20A

This mutation was first described by Akari et al. (Akari et al., 2000). M20A eliminated the ability of Nef to downregulate MHCI in infected CEM cells, but had no effect on CD4 downregulation or the infectivity of HIV-1 virions in single-round replication assays (Akari et al., 2000). Subsequently, internalization studies suggested that M20A resulted in enhanced recycling of MHCI at the plasma membrane (Blagoveshchenskaya et al., 2002). This effect occurred despite there being no reduction of MHCI levels on the cell surface. Since studies of the impact of Nef on MHCI internalization involve lengthy protocols to

allow for Nef expression, the results obtained are comparisons between two different steady state conditions. Effectively, the comparison is between a steady state in which Nef is present and MHCI plasma membrane levels are low to one in which Nef is absent or mutated and MHCI plasma membrane levels are high. For this reason, the transition between steady states cannot be studied. This is in contrast to the seminal studies of Marsh and Pelchen-Matthews in which PMA induced CD4 downregulation was investigated. In this latter case the rapid action of the membrane soluble PMA on CD4 made it possible to describe the kinetics of the shift between steady states from the basal state to the altered state of PMA induced CD4 downregulation (Marsh and Pelchen-Matthews, 1996).

In an alternate approach to studying Nef, Walk et al developed a chimera with Nef fused at its C-terminus to the hormone-binding domain of the estrogen receptor (ER) (Walk et al., 2001). The linking of Nef within to ER resulted in inhibition of all Nef activities, as a result of steric hindrance from cellular proteins binding to ER (Pritchard et al., 1995; Klippel et al., 1998; Kohn et al., 1998). 4-hydroxytamoxifen addition to cells expressing Nef-ER rapidly relieved this inhibition. With this system Walk et al could activate Nef and follow the time course of several Nef effects. They observed that Nef induces PAK2 activation within 15 minutes, CD4 downregulation in 2 hours, but required one day to begin to downregulate MHCI and two days for the full effect.

As described above, two mechanisms have been proposed for MHCI downregulation. First, Nef may block the transit of newly synthesized MHCI to the plasma membrane (Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). Such a block could be sufficient for a downregulation process that is slow in developing, making M20 a potentially critical residue. This mechanism appears to be consistent with a delayed reduction in MHCI downregulation (Dikeakos et al.; Walk et al., 2001). A block in MHCI transiting to the plasma membrane may be advantageous to the virus, since HIV-1 antigens would not be delivered to the surface of the infected cell (Yi et al.).

In addition, a complex mechanism for the activation of PI3K involves Nef binding to PACS-2 leading to the elevated levels of PIP_3 (Dikeakos et al.; Hung et al., 2007; Atkins et al., 2008; Youker et al., 2009). This elevation of PIP_3 is proposed to recruit an ARF6-GEF which in turn activates ARF6 to actively internalize plasma membrane MHCI. Though it is not clear that this process reduces the level of MHCI on the cell surface, a recent report suggests an alternate scenario for blocking killing by cytotoxic T cells without downregulation of MHCI from the cell surface. Dis-regulated MHCI trafficking may interfere with presentation of HIV-1 antigens by MHCI (Yi et al.). This concept could be tested by the same in vitro assays that demonstrated that MHCI downregulation prevents killing of infected cells by cytoxic T cells except that the infectious virus would encode NefM20A (Collins et al., 1998; Yang et al., 2002).

The effort directed to the M20A mutation has been minimal relative to other mutations that negate MHCI downregulation. In fact, it remains to be determined if MHCI downregulation strictly requires a methione at position 20. Isoleucine is common at position 20 in subtype C Nefs and dominant in subtype E Nefs (O'Neill et al., 2006a; O'Neill et al., 2006b). Thus, it is important to define the structural requirement at residue 20 for MHCI downregulation.

AAAA

The designation AAAA refers to a quadruple mutation of Nef in which a string of four glutamates at positions 62–65 are all mutated to alanine. This mutation prevents MHCI downregulation but is not specific. Though the mutant protein has no effect on CD4 downregulation, it is 70% defective for PAK2 activation and 80% defective for enhancement of virion infectivity (Baugh et al., 2008). It is noteworthy that mutations with

only two of the four glutamates replaced by alanines, all exhibit near full activity to downregulate MHCI. In fact, it requires replacing three of the four glutamates with alanine to attenuate MHCI downregulation by 50% (Baugh et al., 2008). Therefore, the quadruple mutation, AAAA, has a synthetic phenotype making mechanistic interpretation difficult. The specificity of the tetraglutamate segment in Nef was further tested with the DDDD mutation. The DDDD mutation in Nef had a greater impact on Pak2 activation (60% inhibition) than it did on MHCI downregulation (no effect) indicating the lack of a specific role in MHCI downregulation (Baugh et al., 2008). These results demonstrate that conservative mutations such as glutamate to aspartate instead of alanine and mutating two or three glutamates to alanine instead of all four can alter mechanistic interpretation. From this evidence it appears that Nef does not contain a functional acidic cluster. Atkins et al have reported siRNA knockdown experiments supporting roles for PACS-1and PACS-2 in MHCI downregulation (Atkins et al., 2008). An alternate interpretation is that targeting PACS-1 or PACS-2 has general effects on intracellular trafficking and an indirect effect on MHCI downregulation. Lubben et al have published conflicting knockdown data (Hung et al., 2007; Lubben et al., 2007).

A subsequent proposal for the role of the four glutamates consistent with weak and non-specific binding is the stabilization of the ternary complex of Nef, the MHCI cytoplasmic tail, and AP-1 (Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). This latter model was tested by insertion of prolines directly before, in the middle, and directly after the four glutamates. It was observed that the extent of MHCI downregulation, observed for these three mutant Nefs, was only reduced by 30% or less. Therefore, it is likely that the four glutamates function as a flexible loop instead of a highly specific protein-protein interface (Baugh et al., 2008).

The AAAA quadruple mutation has not been amenable to straightforward mechanistic interpretation. We suggest that other mutations be investigated as better approaches to understanding MHCI downregulation. The previously discussed M20A which appears to be specific for MHCI downregulation needs to be carefully investigated. Other striking mutations that abrogate MHCI downregulation that are discussed below are D123E and P78 (Yamada et al., 2003; O'Neill et al., 2006b; Atkins et al., 2008; Noviello et al., 2008; Wonderlich et al., 2008; Singh et al., 2009). Unlike the considerable sequence variability of the tetraglutamate segment of Nef, D123 and P78 are 99% conserved in Nefs from HIV-1 subtypes B, C, and E (O'Neill et al., 2006a; O'Neill et al., 2006b).

AXXA

The designation, AXXA, refers to a double mutation of critical prolines in the SH3 binding domain of Nef (P72A/P75A). The full sequence of the Nef interface with the planar, hydrophobic surface of SH3 domains is PQVPLR (Lee et al., 1996; Arold et al., 1997). These six amino acids reside in a polyproline type II helix (PPII) and conform to the canonical sequence for class II SH3 ligands, PXXPX(K/R) (Li, 2005). AXXA refers to AQVALR which resides in the larger sequence, PVRPQVPLRP, (amino acids, 69–78). The crystal structure of Nef bound to Fyn and FynR96I SH3 domains demonstrated that residues P72, V74, P75, and R77 are critical for the Nef-SH3 domain interaction (Lee et al., 1996; Arold et al., 1997). Manninen et al. have mutated these four residues singly and tested the mutant Nefs for their ability to bind the Hck SH3 domain in vitro (Manninen et al., 1998). Mutation of P72 to alanine gave a Nef with a 16 fold reduction in K_D relative to wild type Nef (0.26 μM to 4.18 μM). Similarly, the P75A gave a 20 fold reduction in K_D. The double mutation which is AXXA, resulted in no binding at all. The drastic mutations of V74D and R77E also obliterated binding (Manninen et al., 1998).

Li has suggested that PPII class II ligands with either of the prolines or the flanking arginine mutated should have SH3 binding greatly diminished (Li, 2005). Thus, three obvious mutations for Nef to test for a SH3 domain protein requirement in a given Nef function are the single mutations, P72A, P75A, and R77A. In the case of PAK2 activation, this has been done for P72A, P75A and R77E and indeed all of these singly mutated Nefs do completely lose their PAK2 activation capacity (Wiskerchen and Cheng-Mayer, 1996; Manninen et al., 1998). This evidence strongly suggests that there is a SH3 domain protein required for the activation of PAK2 by Nef.

It has also been reported that the AXXA mutation blocks the signaling pathway of MHCI downregulation by preventing Nef from interacting with Hck, Lyn or Src (Dikeakos et al.). Since the Nef-Hck interaction has been described in great detail it is expected that P72A, P75A, R77A, but not P78A should cripple the MHCI signaling pathway (Dikeakos et al.; Lee et al., 1996; Arold et al., 1997; Manninen et al., 1998). Doubt has been cast on a role for a SH3 binding protein in MHCI downregulation by studies with the CEM T cell line by comparing the AXXA mutation with the single mutations P72A, P75A, and P78A (Yamada et al., 2003; Casartelli et al., 2006). It should be noted that CEM cells exhibit M20 dependent downregulation of MHCI, but only the stoichiometric pathway is operative because of constitutive high levels of PIP_3 masks the signaling pathway (Dikeakos et al.; Freeburn et al., 2002). Interestingly, CEM cells do not exhibit constitutively low cell surface levels of MHCI nor is the extent of Nef downregulation of MHCI in these cells significantly impacted by expression of the PIP_3 phosphatase, PTEN (Dikeakos et al.). Although mutation of either P72A or P75A would be expected to cripple Nef binding to a SH3 domain, these singly mutated Nefs only exhibit a partial loss of function (Yamada et al., 2003). Conversely, P78 which lies outside of the expected Nef-SH3 interaction domain would not be expected to have a significant role in Nef-SH3 binding (Lee et al., 1996). Nonetheless, the Nef mutant P78A fully abrogates MHCI downregulation (Yamada et al., 2003; Casartelli et al., 2006). Therefore, it is likely that the AXXA mutation is blocking the stoichiometric pathway for MHCI downregulation by a different mechanism that it blocks the signaling pathway for MHCI internalization. The interpretation based on the P78 mutant that an SH3 domain protein is not involved in MHCI downregulation per se would be confirmed if a R77 mutation of Nef had no effect on MHCI downregulation. For these reasons P72A, P75A, P78A and R77A mutations are preferred to the AXXA mutation for investigating the mechanism of MHCI downregulation.

The AXXA mutation also blocks Nefs ability to enhance virion infectivity though one report found only partial effects (Goldsmith et al., 1995; Pizzato et al., 2007). Targeting of B cells by long range conduits by Nef is partially blocked by AXXA (Xu et al., 2009). Fine structure mutational analyses for these Nef functions are needed. Involvement of a Nef-SH3 domain interaction would also be strongly indicated if NefR77A failed to exhibit these functions. Careful mutational analysis of MHCI downregulation, PAK2 activation, enhancement of virion infectivity, and class switching inhibition will indicate which of these Nef functions require SH3 domain proteins. The finding of Wiskerchen and Cheng-Mayer that the P69A mutant of Nef does not activate PAK2 suggests an SH3 domain protein different from Hck is involved (Wiskerchen and Cheng-Mayer, 1996). To address the question of how many different SH3 domain proteins other than Hck interact with Nef for PAK2 activation and other Nef activities requires additional mutational analyses. While all SH3 domains that bind PPII class II ligands require PXXPX(R/K), it is the non-proline residues within PQVPLR and residues that flank PQVPLR that are expected to determine binding specificity (Zarrinpar et al., 2003). Mutation of these residues may reveal distinct interfaces for the several Nef functions that do require an SH3 binding domain protein.

R106A

R106 is a highly conserved residue that is necessary for several Nef activities. When Nef is mutated at R106 to alanine multiple defects are observed. This Nef mutant is fully defective for PAK2 activation and enhancement of infectivity, partially defective for CD4 downregulation, and for MHCI downregulation (Manninen et al., 1998; O'Neill et al., 2006b). The defect in CD4 downregulation is consistent with the report that R106 is one of eight residues that interact with the cytoplasmic tail of CD4 in vitro (Grzesiek et al., 1996a). The extreme mutation R106L is fully defective for MHCI and CD4 downregulation (O'Neill et al., 2006b). An early report that the R106L mutation prevented Nef from activating PAK2 was interpreted as this arginine being critical for PAK2 activation (Sawai et al., 1995). Given the multiple defects exhibited by the R106L mutant, an alternate hypothesis is that there is a general disruption of Nef structure. Based on the 99% conservation of R106, we tested the conservative mutation R106K as a more stringent test of the significance of this arginine for several Nef functions. Similar to the R106A mutant, the R106K mutation was partially functional for CD4 downregulation and for MHCI downregulation. In contrast to the R106A mutant which is inactive for PAK2 activation and enhancement of virion infectivity R106K is partially active for PAK2 activation and fully functional for enhancement of infectivity (O'Neill et al., 2006b). Therefore, there does not appear to be any known Nef function with a stringent structural requirement for arginine at 106. These results clearly illustrate that mechanistic interpretations based on drastic mutations like R to L (BLOSUM62, score of −2) are likely to not be representative of the conclusions based on conservative mutations like R to K (BLOSUM62, score +2) (Henikoff and Henikoff, 1992). One possibility suggested by these results is that the critical role of R106 has not been discovered.

LLAA

This designation refers to the mutation of leucines 164 and 165 to alanines. These leucines are in the Nef dileucine motif which is ENNSLL (amino acids 160–165). It has been observed that LLAA is defective for CD4 downregulation, the enhancement of virion infectivity, inhibition of class switching, but not MHCI downregulation. The LLAA mutation also prevents the Nef induced reduction of the endocytic recycling compartment (ERC) (Madrid et al., 2005; Coleman et al., 2006; Pizzato et al., 2007; Lindwasser et al., 2008; daSilva et al., 2009; Laguette et al., 2009b; Xu et al., 2009). The ERC is composed of narrow diameter tubules derived from sorting endosomes. These structures represent two distinct organelles that are jointly designated as early endosomes (Gruenberg and Maxfield, 1995; Maxfield and McGraw, 2004).

As for the mechanism of CD4 downregulation, Nef engages AP-2 through leucines 164 and 165 and the diacidic motif (174 and 175) to increase CD4 internalization as described above. By a separate mechanism, the dileucine-dependent Nef-induced disruption of the ERC appears to reduce the cell surface levels of the transferrin receptor (Madrid et al., 2005). This last Nef phenotype is also lost with the E160A mutation. The glutamate at 160 is important for canonical interactions of the dileucine motif, (E/D)XXXL(L/I) (Bonifacino and Traub, 2003). The in vitro binding of Nef to AP-1 is also dependent on the presence of E160 (Coleman et al., 2006). Therefore, it is significant that NefE160A does downregulate CD4 (Coleman et al., 2006). These results indicate that the LLAA mutation by itself is insufficiently precise to distinguish between canonical or non-canonical interactions. At a minimum, the E160A mutation is also needed. The enhancement of infectivity and inhibition of class switching are lost with the LLAA mutation of Nef, but it is not known if its mechanism overlaps with other dileucine motif mechanisms (Madrid et al., 2005; Pizzato et al., 2007; Xu et al., 2009).

EDAA or DDAA

There are two related mutations that convert the acidic residues at Nef positions 174 and 175 to alanines. Position 174 is highly conserved in the sense that 98% of HIV-1 Nefs have one of the acidic amino acids, either aspartate or glutamate. Therefore, the designation EDAA or DDAA depends on the Nef allele that is being mutated. Position 175 is strictly aspartate (O'Neill et al., 2006b). The diacidic motif is necessary for CD4 downregulation, but not for MHCI downregulation or PAK2 activation (Greenberg et al., 1998; Haller et al., 2007). As previously discussed, Nef mediates CD4 downregulation by forming a ternary complex with the cytoplasmic tail of CD4 and AP-2 (Lindwasser et al., 2008; Chaudhuri et al., 2009). Unlike the other signature mutations discussed above, it is known that a single conservative mutation D175E gives the same fully defective phenotype as the double alanine mutation (Lindwasser et al., 2008). This latter finding is of particular importance since it allows for a stringent test for the relevance of the non-canonical binding of Nef to AP-2. Lindwasser et al. made the remarkable observation that the D175E mutant displayed a severely reduced binding to the AP-2 γ-σ1 hemicomplex in the yeast three hybrid system (Y3H). The K_D for the wild type interaction was found to be 6 μM by SPR spectroscopy (Lindwasser et al., 2008). The high degree of specificity for D175 is emphasized by the BLOSUM62 score of +2 for D to E. Mutation of D to A has a score of −2 (Henikoff and Henikoff, 1992). These results dramatically confirm the proposed mechanism for Nef-mediated CD4 downregulation (Lindwasser et al., 2008). Two other proteins have been reported to bind to Nef but not to Nef with the EDAA or DDAA mutations (Hodge et al., 1998; Lu et al., 1998; Geyer et al., 2002). The K_D's for these interactions have not been reported, and it remains to be determined if these proteins bind to Nef with the D175E mutation. Finally, the fact that the EDAA mutation is phenotypically the same as D175E, is potentially useful for experimental systems where HIV-1 is replicating and capable of reverting point mutations (Melkus et al., 2006). The double alanine mutation would be a highly specific defect, but refractory to reversion. The apparent equivalency of the phenotype for the standard double alanine mutation and the highly conservative mutation is unique for EDAA and DDAA. For AAAA, AXXA, and R106A, phenotypic equivalence with conservative mutations is not the case and for M20A and for LLAA it has not been tested.

F191I or F191A

F191 is critical for PAK2 activation for subtype B Nefs (Foster et al., 2001; O'Neill et al., 2006b). As previously discussed subtype E Nefs have arginine at position 191 and introduction of F191R into the subtype B Nef from HIV-1_{SF2} debilitates PAK2 activation. The finding of this highly unusual functional difference between HIV-1 subtypes led to extensive mutational analysis of F191 function in the HIV-1 subtype B SF2Nef. All replacements for F191 tested (I, Y, L, R, H, and A) were found to be inactive (O'Neill et al., 2006b; Rauch et al., 2008). The F191I mutation is the most studied of the six mutations. It is remarkably specific for PAK2 activation with little or no effect on CD4 downregulation, MHCI downregulation, or enhancement of virion infectivity (Foster et al., 2001; O'Neill et al., 2006b).

The role for F191 in PAK2 activation has not been elucidated though it has been suggested that Vav is involved. On basis of lipid raft experiments it was suggested that F191 was part of the Nef interface that interacts with Vav (Rauch et al., 2008). This finding appears to contradict an earlier finding that the second SH3 domain of Vav interacts with Nef but not the AXXA mutant of Nef (Fackler et al., 1999). In addition, siRNA experiments knocking down either Vav1, Vav2, or ELMO all gave approximately 30% reductions in PAK2 activation. Therefore, indirect effects must be considered as an alternate explanation, and the role of Vav remains unclear. It has also been reported that Nef strongly induces

phosphorylation (inhibitory) of cofilin by a PAK2 activation mechanism (Stolp et al., 2009). It is noteworthy that the inhibitory phosphorylation site on cofilin is not in a PAK2 phosphorylation site context (Tuazon et al., 1997). Stolp et al. demonstrated that purified PAK2 in the presence or absence of Nef does not significantly phosphorylate cofilin. This suggests that Nef does not directly bind to PAK2. However, in this report anti-Nef immunoprecipitates with bound, activated PAK2 from lysates of Nef expressing cells were able to strongly phosphorylate cofilin in an F191-dependent manner (Stolp et al., 2009). One explanation for these results is that Nef forms a multi-protein comlex which alters the substrate specificity of PAK2.

D123E

This is a non-standard mutation, but representative of a large number of published mutations of aspartate at position 123, including A, E, G. L, V, and R (Kwak et al.; Cohen et al., 2000; Liu et al., 2000; Swigut et al., 2000; Arold and Baur, 2001; Williams et al., 2005; O'Neill et al., 2006b). Mutations of this residue are all multi-defective. For example D123G is completely defective for CD4 downregulation, MHCI downregulation, and enhancement of infectivity (Liu et al., 2000). Moreover, the conservative D123E mutant and the drastic D123R mutant have the exact same phenotype (Kwak et al.; O'Neill et al., 2006b). These remarkable findings in which D123E (BLOSUM62 score, +2) has the exact same phenotype as mutations with BLOSUM62 scores ranging from −1 to −4, implies a unique role for D123 in Nef function. The complexity of this phenotype has led to speculation that D123 is part of a dimer interface (Arold et al., 2000; Liu et al., 2000; Poe and Smithgall, 2009). Poe and Smithgall have suggested that a crystallization interface models the structure of Nef dimers in cells (Poe and Smithgall, 2009). The suggested relevant Nef amino acids are R105, I109, L112, Y115, P121, and D123. In this model a roughly anti-parallel orientation of the interface consists of hydrophobic interactions between the internal amino acids and salt bridges between R105 and D123.

That Nef dimerizes is well established. When cell free extracts from Nef expressing cells are analyzed by SDS/PAGE Nef resolves into a major monomeric form and a minor dimeric form (Kwak et al.; Kienzle et al., 1993; Zazopoulos and Haseltine, 1993). It is clear that the linkage between the dimeric Nefs is through cystine as SDS/PAGE analysis under reducing conditions yields only monomer. Alternately, some investigators have used a CD8Nef chimera with Nef replacing the cytoplasmic domain of CD8. Here there is a discrepancy in the literature. Several reports find that SDS/PAGE analysis under reducing conditions gives only the monomeric chimera, but Liu, et al. find a significant species of CD8Nef dimer (Sawai et al., 1994; Sawai et al., 1995; Baur et al., 1997; Bresnahan et al., 1998; Lu et al., 1998; Erdtmann et al., 2000; Liu et al., 2000). Analyzing the CD8Nef chimera under reducing conditions is critical because the extracellular domains of CD8 also form dimers that are cross-linked by cystine (Boursier et al., 1993). Therefore, the nature of the reported linkage between the Nef moieties of CD8Nef dimers reported by Liu et al. is unknown. Interpretation of the result of Liu et al as evidence for dimers requires clarification of the discrepancies in the literature and extening of the result to native Nef.

Poe and Smithgall have reported that mutation of D123 disrupts the proposed R105–D123 dimeric interface as determined by bimolecular fluoresecence complementation (BiFC). This experimental approach requires two Nef chimeras. In one chimera the last five amino acids of Nef were replaced by amino acids 2–154 of YFP and the second Nef was linked near the C-terminus of Nef to amino acids 154–238 of YFP. Co-expression of the two Nef chimeras in cells lines yields a fluorescent signal indicative of Nef dimerization. Mutation of the two Nef moieties at D123 or its salt bridge partner, R105 diminishes the fluorescent signal, but the extent is dependent on which mutation is present. The D123N, D123A,

D123V, R105E, and R105E/R106E mutations decrease BiFC by 97%, 70%, 53%, 50%, and 71% respectively, but all of the aspartate mutations and the double arginine mutation are completely defective for CD4 downregulation and HIV-1 replication in modified U87MG astroglioma cells. The phenotype of the R105E mutant was not tested (Poe and Smithgall, 2009). Therefore, it would appear that a 50% diminution of the BiFC dimers corresponds to complete phenotypic disruption. In contrast, Kwak et al, assayed for the presence of dimers by intracellular cross-linking by formaldehyde and found no impact of mutating aspartate-123 to arginine on the level of Nef dimers. This drastic mutation would turn both proposed salt bridges in the model of Poe and Smithgall into sites of charge repulsion (Poe and Smithgall, 2009). One explanation of this discrepancy regarding the role of D123 in dimerization is that the dimers that are being detected by BiFC and formaldehyde cross-linking are different (Kwak et al.; Poe and Smithgall, 2009). Given that the SF2Nef used by Kwak et al has ten lysines it is likely that this approach will detect multiple Nef dimers if present. Further, studies of HIV-1Nef and SIV_{MAC239}Nef homodimerization and heterodimerization indicate that it is likely that Nef dimerizes through multiple interfaces (Kwak et al.). The BiFC approach requires that Nef dimerizes in such a way that the segments of YFP are positioned appropriately to fluoresce and may have a limited capacity to detect different species of Nef dimers. If BiFC is detecting a subpopulation of Nef dimeric forms then impact of mutating D123 on dimerization may vary with the different residues incorporated, even though all D123 mutations have a global impact on multiple Nef phenotypes. In other words, it is not clear that Nef dimers that depend on D123 are responsible for the complex phenotype of mutating D123. This implies a different mechanism for the defect that results from mutating D123 on dimerization versus the mechanism for the distinct multi-defective D123 mutant phenotype. It is also unclear why the R105E and R105E/R106E mutations do not completely disrupt dimerization as the pair of D123/R105 salt bridges in the dimerization model are converted to D123/E105.

The role of Nef dimerization in the activation of Hck was investigated by Ye et al (Ye et al., 2004). These investigators employed a similar approach to the previously described report of Walk et al by making a Nef/estrogen receptor hormone binding domain chimera. Y et al. observed that enforced dimerization of Nef activated Hck to levels in several fold in excess of that observed for wild type Nef. This result strongly supports a role for a dimeric form of intracellular Nef that is present at a low level in the absence of enforced dimerization (Ye et al., 2004). However, Walk et al. did not observe an enhancement in the Nef functions of CD4 downregulation, PAK2 activation, or MHCI downregulation (Walk et al., 2001). It would be expected that D123E would fail to block the enhanced Hck activation by the Nef/estrogen receptor hormone binding domain chimera, but the results of Walk et al suggest that this conservative mutation would block CD4 downregulation, MHCI downregulation, and PAK2 activation.

Discussion

Much of the progress in the Nef field over the last twenty years has been based on analysis of the standard mutations discussed above. With the likely exceptions of EDAA and DDAA, all of the standard mutations either have been demonstrated to exhibit relatively severe phenotypes compared to conservative mutations or have not been tested in this regard. The generation of complex phenotypes has a limited usefulness for mechanistic analysis. That mutations like D123 to A, G, L, V, and R are multi-defective is not a compelling demonstration of a unique role for D123. Conversely, the multiply defective phenotype of the conservative mutation D123E is strongly suggestive of a unique role for D123 in Nef function. It should also be noted that it is important to test highly conserved residues since poorly conserved residues like R105 are not expected to be involved in highly specific interactions at protein/protein interfaces.

In general, we suggest a reappraisal of the standard mutations by making replacements less disruptive than alanine (Table I). Exceptions would be C, S, G, and P for which alanine would be expected to be minimally disruptive. A clear example is the considerably reduced impact of mutating the 99% conserved R106 to lysine instead of the multiply defective R106A or fully defective R106L. The lack of a definitive phenotype for R106K leaves unexplained the fact that lysine is essentially excluded at Nef position 106 (O'Neill et al., 2006b). One possibility is a recently reported role for R106 in Nef dimerization (Kwak et al.). Further, single mutations are preferable to multiple mutations. For example, P72A and P75A are less disruptive than the standard double mutation, AXXA for MHCI downregulation. Just as important for mechanistic interpretation, these single mutations are equally disruptive for PAK2 activation as the Nef AXXA mutation which strongly indicates the involvement of an SH3 domain protein in this Nef function (Manninen et al., 1998).

It is increasingly evident that Nef has evolved into a patchwork of overlapping effector interfaces that is somehow held together structurally. To explore this concept, we asked the question if protein interface prediction could suggest possible arrangements of interacting patches on the surface. PPI-Pred analysis gave three separate patches indicated as here red, green and blue (Figure 1A). The gray represents Nef surface that was not selected by the program. It includes the deletion of the internal loop which may disrupt the analysis. Details of the analysis is given in the Figure Legend.

The residues that make up each of the patches are indicated in Figure 1B. There are three residues that are in two of the three patches: P78, H116, and and F121. P78 and F121 are 99% conserved in subtype B Nefs. Position 116 is highly conserved as H or N with 99% of subtype B Nefs having one or the other. Interactions between the three patches are suggested by the sharing of amino acid residues.

The significance of the patches is enhanced by the fact that three previously proposed Nef interfaces each segregate to one of the three predicted patches. The recently proposed dimerization interface for HIV-1 Nef generated by ClusPro 2.0 (http://clus.bu.edu) is highly represented in the red patch (Kwak et al.). The difference between PPI-Pred and ClusPro is that ClusPro samples the interacting surfaces resulting from the random juxtaposition of two Nef molecules. PPI-Pred evaluates the surface of a single Nef for likely attributes of a protein-protein interface. The thirteen residues are shared between the PPI-Pred generated red patch and the ClusPro generated interface are indicated by astericks. The blue interface contains five residues (*) demonstrated to be involved in the binding of Nef to Fyn by X-ray crystallography (Lee et al., 1996; Arold et al., 1997). The green patch contains two residues suggested to be part of the putative Nef interface and D123 that are important for PAK2 activation (Agopian et al., 2006; O'Neill et al., 2006b). It has also been proposed that this surface binds to Vav (Rauch et al., 2008).

Poe and Smithgall proposed that six residues from one of the Nef-Nef interfaces in hexagonal crystals are important in Nef dimerization (Poe and Smithgall, 2009). Five were selected by PPI-Pred: I109 and L112 are in the red patch; D123 is in the green patch; Y115 and F121 are in the blue patch. The poorly conserved R105 was not included in by PPI-Pred analysis. Clearly, this crystallization interface presents a very different model than that obtained in silico. A better correspondence exists for a crystal interface found in cubic Nef crystals. Four of the five residues in this interface that are at least 90% conserved are present in the green patch: F139, F191, H193, and R196. All three types of predictions based on crystal interfaces, ClusPro, or PPI-Pred merely represent hypotheses. Only careful mutational analysis will reveal the true structure-function relationships for this wondrously complex protein.

Two decades of mutational analysis has slowly accumulated evidence of multiple effector domains on the surface of Nef. These domains cover a considerable portion of the available surface, and it is likely that future work will define additional domains to include a high percentage of Nef's surface. As evidence accumulates structure-function maps of Nef will emerge that are sufficiently detailed to allow deductions regarding complex Nef interactions with host cell proteins. Beyond this structural complexity it has also been proposed that Nef exhibits allosteric properties (Trible et al., 2007). Although the complexity and compactness of Nef makes it a difficult target of study, the field appears to now be poised to describe the mechanisms of Nef's multiple activities. This will greatly facilitate the possible development of drugs for therapeutic intervention by inhibiting Nef. The region surrounding D123 may provide the best chance for developing drug-like molecules that will disrupt multiple Nef functions, including CD4 and MHCI downregulation.

Acknowledgments

This work was supported by grant AI33331 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, USA and UNC CFAR P30 AI504410.

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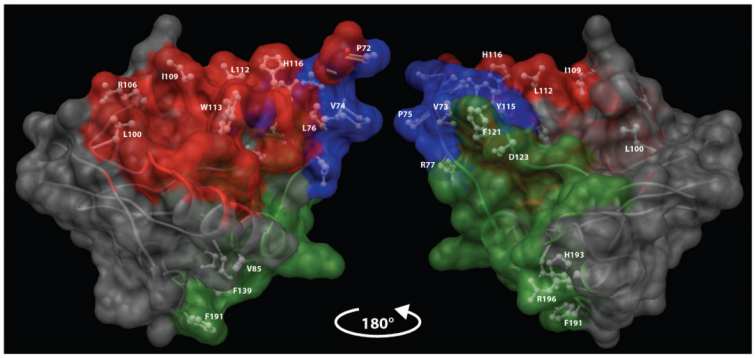
A.



B.

Highest Probability Patch	Second Highest Patch	Third Highest Patch
71	P78	* P72
L87	M79	* Q73
F90	T80	* V74
* L91	Y81	* P75
* K94	K82	L76
* G95	A83	* R77
* G96	* V85	P78
* L97	F121	Y115
* L100	* D123	H116
* R106	W124	F121
* D108	N126	P122
* I109	L137	
L110	T138	
* L112	F139	
* W113	A190	
* H116	* F191	
T117	H193	
Q118	R196	
* W183	Y202	
	F203	

Figure 1. PPI-Pred analysis of Nef

(A) Three dimensional representation of Nef (PDB 1AVZ, Chain B) highlighting consensus regions of predicted protein-protein interaction resulting from PPI-Pred analysis. The patch analysis returns three regions exhibiting properties of protein-protein interfaces ranked by probability. All five Nef molecules from three separate crystal structures (PDBs 1AVZ, 1AVV and 1EFN) were analyzed. In addition, three individual models from the Nef NMR structure, 2NEF, were analyzed (Grzesiek et al., 1996b; Lee et al., 1996; Arold et al., 1997; Grzesiek et al., 1997; Trible et al., 2007). The three models were selected by having the lowest, highest or median RMS scored when aligned with the 1AVZ, Chain 1 crystal structure. Overlapping regions identified in each analysis were compared, and consensus residues were identified by occurrence in a specific region in at least four of the eight structures analyzed. The consensus regions were mapped onto 1AVZ, Chain B to be able to show residues 71, 72, and 73, which are missing in 1AVZ, Chain A. Residues of interest from biochemical data are shown in white ball and stick under a transparent surface. Residues of interest in the red region-L100, R106, I109, L112, W113, H116- correspond to six of eight residues identified in homodimerization docking experiments by ClusPro analysis. P72, Q73, V74, P75, L76, and R77 in the blue region represent the six residues in the proline helix of the Nef SH3 binding domain. Two residues, V85 and F191, of the green region are two of the four residues of a proposed protein interaction interface critical for PAK2 activation. F139, F191, H193, and R196 are found in a crystallization interface in cubic crystals (Arold et al., 2000).

(B) Residues of each region are listed. A few residues are common between the regions. These are P78, H116 and F121.

Table I

Non-negative BLOSUM62 substitution matrix values

Residue	3	2	1	0
C				A
S			T, A, N	G, D, E, Q, K
T			S	A, N, V
A			S	C, T, G, V
G				S, A, N
N			S, D, H	T, G, E, Q, R, K
D		E	N	S, Q
E		D, Q	K	S, N, H, R
Q		E	R, K	S, N, D, H, M
H		Y	N	E, Q, R
R		K	Q	N, E, H
K		R	E, Q	S, N
M		L	I, V	Q, F
I	V	L	M	F
L		M, I	V	F
V	I		M, L	T, A
F	Y		W	M, I, L
Y	F	H, W		
W		Y	F	

Nineteen amino acids are listed in the left column. Amino acid replacements that are relatively common in protein evolution are given in the columns labeled 3, 2, 1, and 0 with a value of 3 representing replacements that are much more common than average. All negative substitution values represent relatively rare replacements, and are not listed. Proline is not given as all replacements of proline have negative scores. (Henikoff and Henikoff, 1992).

It should be noted that there is no Nef involvement at this stage and according this pathway is described as the signaling pathway. Though it is proposed that the signaling pathway brings about enhanced MHCI internalization, the mechanism is not sufficient to necessarily downregulated MHCI from the plasma membrane. In fact NefM20A has been reported to result in increased rates of recycling of cell surface MHCI even though this Nef mutant fails to reduce the amount of MHCI on the cell surface (Blagoveshchenskaya et al., 2002).