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review

Structure–function relationships in HIV-1 Nef

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The accessory Nef protein of HIV and SIV is essential for viral pathogenesis, yet it is perplexing in its multitude of molecular functions. In this review we analyse the structure–function relationships of motifs recently proposed to play roles in aspects of Nef modification, signalling and trafficking, and thereby to impinge on the ability of the virus to survive in, and to manipulate, its cellular host. Based on the full-length structure assembly of HIV Nef, we correlate surface accessibility with secondary structure elements and sequence conservation. Motifs involved in Nef-mediated CD4 and MHC I down-regulation are located in flexible regions of Nef, suggesting that the formation of the transient trafficking complexes involved in these processes depends on the recognition of primary sequences. In contrast, the interaction sites for signalling molecules that contain SH3 domains or the p21-activated kinases are associated with the well folded core domain, suggesting the recognition of highly structured protein surfaces.

Introduction

The functions of the 27–35 kDa myristoylated HIV/SIV Nef protein are only poorly understood although the protein is of great importance for the pathogenesis of the primate lentiviruses. This lack of understanding is reflected in the plethora of mutants and deletion constructs of Nef that have been created in attempts to elucidate its function (see for example Guy *et al.*, 1990; Aiken *et al.*, 1996; Hua and Cullen, 1997; Kim *et al.*, 1999). A large number of cellular interaction partners has been discovered and the binding sites for some of these proteins have been mapped to distinct locations within Nef. The relevance of the proteins to the pathogenesis of the primate lentiviruses, however, is as unclear as the importance of the individual *in vitro* functions of Nef. Thus, elaborate structure–function analyses of Nef may be required to identify the physiologically relevant interaction sites of Nef, and to provide important clues toward the molecular mechanisms used by the viral protein. Here we present such an analysis, based on the structure assembly of the full-length Nef protein (Geyer and Peterlin, 2001).

A summary of Nef binding proteins for which the binding sites in Nef have been mapped is provided by Table I. The locations of these sequence motifs within the structure are illustrated in Figure 1, and can be correlated with the degree of conservation of the surface and backbone structure as displayed in Figure 2. Many of the Nef mutants in which these motifs are affected have already been characterized functionally. The results of these studies are summarized in Table II, and are grouped according to the following widely accepted *in vitro* functions of Nef: the enhancement of virion infectivity, the support of viral replication in peripheral blood mononuclear cells (PBMC), the activation of the Pak kinase and the downregulation of CD4 and MHC class I molecules from the cell surface. Additionally, the contribution of the respective motifs to full pathogenicity of primate lentiviruses in available *in vivo* models is summarized. These features of the mutants are correlated with surface accessibility, secondary structure formation and sequence conservation. We discuss the binding motifs in terms of their function in Nef modification, signalling and trafficking.

Motifs involved in Nef modification

The N-myristoylation of Nef is required for its association with cellular membranes and is critical for virtually all of its biological activities (Table II). Myristoylated proteins start with the sequence Met-Gly, but the initiating methionine is removed during translation and myristate is amide linked to the glycine. The consensus sequence for the *N*-myristoyl transferase protein substrate is MGxxx(S/T), with an additional preference for lysine or arginine at positions 7 and/or 8 (Resh, 1999). In all 186 Nef sequences analysed, this motif is almost absolutely conserved, with no deviation from glycine and serine at positions 2 and 6, respectively, 99% conservation for a lysine at position 7, and additional 48% conservation for a positively charged residue at

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Table I. Sequence motifs of HIV-1 Nef

Region in NL4-3 Nef	Interacting protein	Reference
Protein modification		
$MGxxxS_{(1)}$	*N*-myristoyl transferase	Geyer *et al*. (1999)
$CAW{\downarrow}LEA_{(55)}$	HIV-1 protease	Freund *et al*. (1994)
Signalling		
$PxxPxR_{(72)}$	SH3 domains of Src family kinases (Hck, Lck); Vav	Saksela *et al*. (1995); Fackler *et al*. (1999)
	TCR ζ	Xu *et al*. (1999)
$RR_{(105)}$	Pak1/2	Renkema *et al*. (1999); Fackler *et al*. (2000)
$DDPxxE_{(174)}$	c-Raf1 kinase	Hodge *et al*. (1998)
Trafficking		
$WL_{(57)}$, $L_{(110)}$	cytoplasmatic tail of CD4	Grzesiek *et al*. (1996)
$EEEE_{(62)}$	PACS-1	Piguet *et al*. (2000)
$FPD_{(121)}$	human thioesterase	Liu *et al*. (2000); Cohen *et al*. (2000)
$EE_{(154)}$	β-COP	Benichou *et al*. (1994); mapping by Piguet *et al*. (1999)
$ExxxLL_{(160)}$	adaptor proteins AP-1/2/3	Bresnahan *et al*. (1998); Craig *et al*. (1998)
	β1 of AP-1	Greenberg *et al*. (1998)
$DD_{(174)}$	V1H	Lu *et al*. (1998)

position 8. This highlights the requirement for membrane localization of Nef for its role in the viral life cycle (Harris, 1995).

Another post-translational modification of Nef results from its specific cleavage by the viral protease within the virion. However, it remains unclear whether this process is of functional relevance (Pandori *et al*., 1996; Welker *et al*., 1996). The HIV-1 protease cleavage site is located between W57 and L58 and determines the modular organization of Nef, separating it into anchor and core domains (Freund *et al*., 1994). The six residues at this site are all very well conserved, particularly tryptophan 57. This residue also marks an important contact to a hydrophobic groove in the core domain, which stabilizes the rather flexible PxxP loop formed by amino acids W57 through T80 (Grzesiek *et al*., 1997). However, the nature of this interaction suggests that protease recognition and cleavage may require greater exposure of this stretch, which could result from a dynamic conformational exchange process.

Motifs involved in signalling

In HIV-1 Nef, the left-handed polyproline helix type II extends over 10 residues, forming a $(Pxx)_4$ [also known as $(PxxP)_3$] sequence cluster. This proline-rich motif mediates the interaction between Nef and signalling molecules such as Hck and Vav, and is central to Nef's ability to induce cellular activation, a function that may be required specifically for the support of viral replication in resting cells (Saksela *et al*., 1995; Fackler *et al*., 1999). Of the four prolines within the helix, only the inner two satisfy the more specific requirement for a bona fide SH3 binding site, defined as the PxφPxR motif, where φ is a hydrophobic amino acid and most often a valine (Lim *et al*., 1994). Based on the two crystal structures of Nef complexed with the Fyn SH3 domain, 12 residues in Nef were identified to form the interface to an SH3 domain (Lee *et al*., 1996; Arold *et al*., 1997). Besides P72, V74, P75 and R77, residues D86, F90, W113 and T117 are also extremely conserved. These amino acids all participate in side-chain interactions with Fyn, explaining the requirement for specific residues at the surface of Nef. Furthermore, residues K82 and I114, which form a salt bridge and participate in hydrophobic interactions, respectively, are both exclusively replaced by their homologous residues, R82 and V114. Finally, Q73 is conserved at 99% and Y120 at 90%, the latter most often being replaced by phenylalanine. These data highlight the importance of the Nef SH3 domain interaction site, which remains highly preserved along its entire interface despite the sequence variations in other parts of the Nef protein (Figure 2).

A peculiarity of the NL4-3 and LAI alleles of Nef is a threonine residue at position 71, which directly precedes the PxφPxR motif. This amino acid is most often found to be an arginine (79%) or the homologous lysine (15%). In fact, R71 also contributes to the SH3 binding interface by forming a hydrogen bond and possibly participating in electrostatic interactions with the SH3 domain (Lee *et al*., 1996), and the NL4-3 and LAI Nef proteins lack this interaction (Arold *et al*., 1997).

The interaction between Nef and a Nef-associated kinase (NAK) is central for cellular activation by Nef, and has been implicated in enhanced viral infectivity and production as well as in disease progression (Sawai *et al*., 1996). However, the importance of this interaction was not confirmed in all of the studies that were performed (Lang *et al*., 1997), and these discrepancies still await clarification. The situation is complicated by the different relative contributions of specific motifs (such as the PxxP) in the Nef proteins from HIV-1 and SIV. Furthermore, activities such as cell activation by Nef may be required for efficient virus spread (and thus pathogenic potential) in some, but not all, physiological scenarios. It was recently suggested

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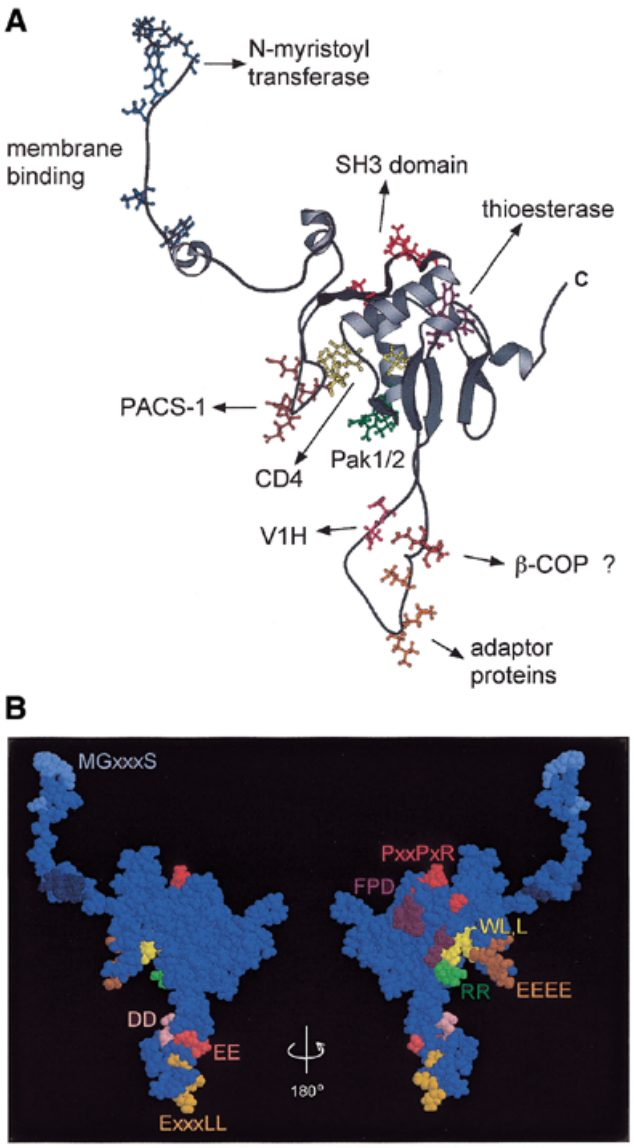

Fig. 1. Location of functional motifs in the HIV-1 Nef structure. (**A**) Functional motifs in HIV-1 Nef (Table II) and their location in the full-length structure as derived from the NMR structure assembly. Binding sites for the known interacting proteins are indicated. (**B**) The van der Waals surface presentation of Nef indicates the accessibility of the interacting residues. The colour coding corresponds to the labelled residues in (A).

that two members of the p21-activated kinase (Pak) family, namely Pak1 and Pak2, can act as NAKs (Renkema *et al.*, 1999; Fackler *et al.*, 2000).

Two arginines at the N-terminus of helix α4 of Nef (R105, R106) are required for its interaction with Pak in co-immunoprecipitation and pull-down assays (Sawai *et al.*, 1995; Fackler *et al.*, 2000). Mutation of these residues to leucines abrogates Nef-induced Pak activation, but it is not yet clear whether the arginines mediate the direct interaction between Nef and Pak. While both residues are exposed on the molecular surface, only R106 is absolutely conserved. At position 105, the positively charged lysine is present most often (68%), with arginine or glutamate taking its place in the remaining homologues (23 and 9%, respectively). The localization of the two positive charges to the N-terminal cap of the helix might reflect a strong impact on helix formation, in which case any mutation here could influence the folding process and, thereby, protein stability (Iafrate *et al.*, 1997). This idea is supported by the partial loss of other biological activities upon mutation of these residues (Table II).

Targeted mutational analysis has identified four other residues that affect Pak binding; these are surface residues located between the SH3 binding site and the RR motif (Manninen *et al.*, 1998). All of these amino acids (P69, L76, L112 and F121) are extremely well conserved (>99%), suggesting that they form a common interaction surface (Figure 2). It should be noted that the PxxP motif itself appears to be required for the activation of Pak via the recruitment of Vav, but does not participate directly in the interaction between Nef and Pak (Fackler *et al.*, 1999, 2000). In addition to these functions in cell activation, Nef has also been reported to inhibit T cell receptor signalling (Iafrate *et al.*, 1997). However, the reasons for these differing results remain to be defined.

Motifs involved in trafficking

To date, six sequence regions of Nef have been described to be involved in the internalization and trafficking of Nef, events that mediate the degradation of CD4 and MHC I. Downregulation of CD4 may prevent superinfection and counteract interference with the viral envelope (Benson *et al.*, 1993; Lama *et al.*, 1999; Ross *et al.*, 1999), while MHC I downregulation helps infected cells to escape the CTL response (Collins *et al.*, 1998). The data on the protein interactions of Nef that facilitate internalization remain, at least in part, controversial. Discrepancies between studies may have arisen due to typically low affinities between proteins that take part in the transient assembly of adaptor complexes that are used in endocytic trafficking (Marsh and McMahon, 1999). Also, many of the proteins involved exhibit poor solubility properties due to membrane localization.

The first of the trafficking interactions to be analysed was that between a 13 amino acid peptide of the cytoplasmic tail of CD4 (aa 407–419), including a critical di-leucine motif at position 413, 414 (Grzesiek *et al.*, 1996), and Nef. The binding affinity of this peptide to N-terminal truncated Nef ($Nef\Delta^{2-39}$) has been determined to ~1 mM resolution by NMR spectroscopy. Mapping of this interaction to the Nef surface delineates a binding region that forms a hydrophobic patch of 10 residues encompassing W57, G96, R106 and I109. Most strongly affected by CD4 binding are the side chains of the W57, L58 and L110 residues, all of which are highly conserved among Nef alleles (Figure 1).

Three of the other motifs that play roles in trafficking have been reported to be involved specifically in Nef internalization. These are located in the C-terminal flexible loop of Nef (148–180), and are fully accessible to interacting proteins. There is a striking symmetry in the formation of the flexible loop. A di-leucine motif (L164, L165) is located at the centre of the loop and is therefore the most highly exposed, while two negatively charged regions (E154, E155 and D174, D175) are equidistant in sequence from the di-leucine motif, and almost equidistant from the preceding and succeeding β-strands, β4 and β5. Together,

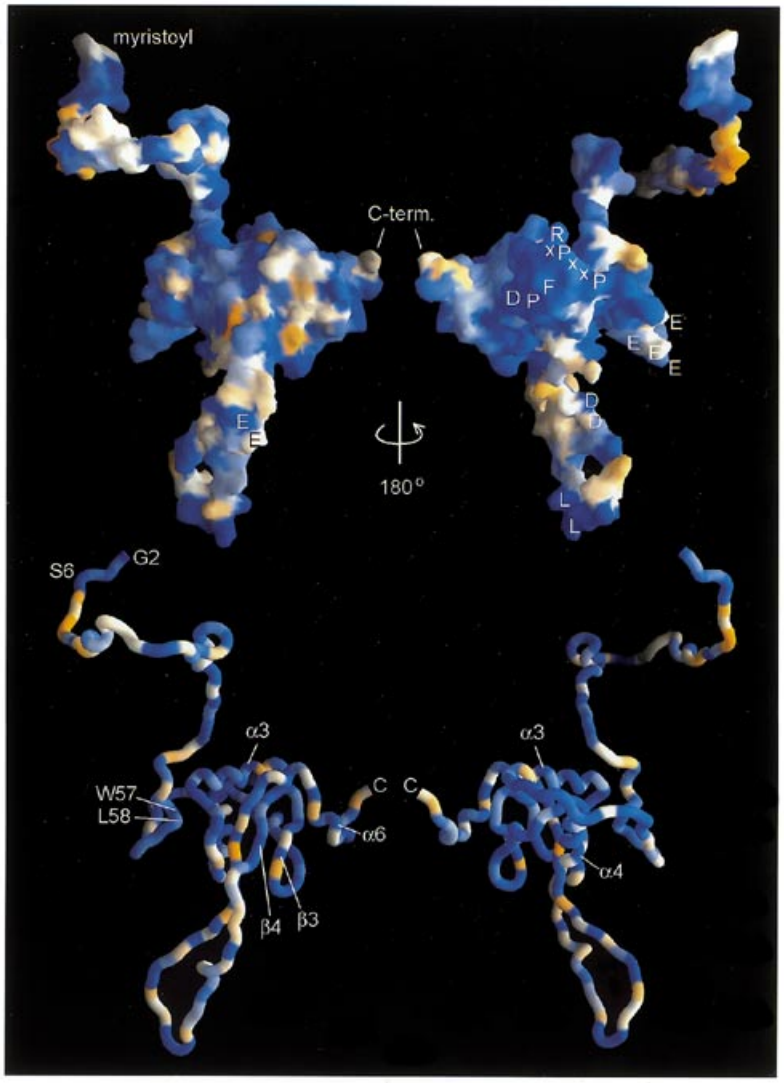

Fig. 2. Sequence conservation superimposed onto the surface and the backbone structure of HIV-1 Nef. The colour coding corresponds to the degree of conservation ranging from 30% (orange) to 84% (white, average conservation) to 100% (blue). Some of the interaction sites listed in Table I are indicated on the structure. The figure was generated with GRASP (Nicholls *et al.*, 1991).

the latter four residues form a negatively charged cluster, seven or six amino acids downstream or upstream, respectively, of the Nef core structure. Of the three recognition sites, the endocytic (E/D)xxxLL signal (di-leucine motif) is best preserved, being absolutely conserved in all sequences. This motif has been shown to be essential for the sorting of Nef into clathrin-coated pits, a process mediated by the interaction with adaptor complexes AP-1 and AP-2 (Bresnahan *et al.*, 1998; Craig *et al.*, 1998; Greenberg *et al.*, 1998). This motif likely binds the β- or μ-subunit of adaptor protein complexes, although its recognition domain has not yet been mapped precisely (Marsh and McMahon, 1999). In the case of the two aspartic acids (D174, D175) succeeding the di-leucine motif, mutation to alanine completely abolishes the ability of Nef to downregulate CD4 (Aiken *et al.*, 1996). The catalytic subunit H of the vacuolar ATPase (V1H) has been shown to interact with this region (Lu *et al.*, 1998), and to lead to Nef internalization by bringing it to the endocytic machinery. The diacidic character of this motif is completely conserved, although the first position may be either an aspartic acid or a glutamic acid residue. The only functional motif of Nef that is not well conserved is the cluster of two glutamic acids (E154, E155) that precedes the di-leucine motif. This diacidic-based motif is also described to function in endocytic trafficking, in this case as a lysosomal targeting signal that binds to β-COP in endosomes (Piguet *et al.*, 1999). However, although even the conservative mutation from glutamic acid to glutamine was reported to abolish the interaction with β-COP, ~20% of all Nef alleles have a lysine at position 155. This naturally occurring charge reversal from EE to EK raises some doubts about the importance of this interaction and the accuracy of the mapping.

Table II. Sequence requirements of HIV-1 Nef for respective functions[a,b]

Residues in Nef_{NL4-3}	Infectivity	PBMC replic.	Pak activation	CD4↓	MHC-I↓	Pathogenicity
$MGxxxS_{(1)}$	+	+	+	+	+	+[c]
$WL_{(57)}$	+/–	+	–	+	–	n.d.
$EEEE_{(62)}$	+/–	+/–	n.d.	–	+	+[c]
$PxxP_{(72)}$	+/–	+	+	+/–	+	+/– [c,d]
$RR_{(105)}$	+/–	n.d.	+	+/–	n.d.	+[c,d]
$FPD_{(121)}$	+	n.d.	n.d.	+	+	n.d.
$EE_{(154)}$	n.d.	n.d.	n.d.	+/–	n.d.	n.d.
$LL_{(164)}$	+	+	n.d.	+	–	n.d.
$DD_{(174)}$	n.d.	n.d.	n.d.	+	–	+[d]

[a]+, required for function; +/–, partially required; –, not required; n.d., not determined.
[b]Requirements were judged based on currently available experimental data. Note that the results for specific mutations may vary depending on *nef* alleles used and the nature of the mutated residues.
[c]The requirement for the various motifs in Nef for pathogenesis was based on experiments with HIV-1 in the SCID/hu mouse model.
[d]Deduced from *in vivo* experiments with SIV. Note that the relative contributions of conserved motifs may differ between Nef proteins from HIV-1 and SIV.

A fifth Nef domain was recently recognized as being involved in trafficking when it was reported that Nef may bind directly to PACS-1 (Piguet *et al.*, 2000), a molecule that controls endosome-to-Golgi trafficking (e.g. of the cellular protein furin and the mannose-6-phosphate receptor). The interaction between Nef and PACS-1, which is supposed to mediate retention of MHC I by Nef in the *trans*-Golgi, was mapped to an acidic cluster of four successive glutamic acids in Nef [$EEEE_{(62)}$]. In fact, this motif is better preserved than it seems at first glance (Geyer and Peterlin, 2001), considering that in most cases (52%) the source of variation is the insertion of a fifth acidic residue (E or D), and the non-conservative insertion of a glycine occurs in only eight out of 186 Nef sequences. Also of note with regard to the conservation of this domain is the fact that the recognition motif for PACS-1 seems to be generally very promiscuous; many proteins to which PACS-1 binds contain additional phosphorylated residues with respect to the defined binding motif (Molloy *et al.*, 1999). Interestingly, HIV-1 Nef lacks potential phosphorylation sites surrounding this motif, while SIV Nef sequences offer an acidic cluster region in the flexible loop [e.g. in the SIV_{mac239} allele: $SDEAQEDEE_{(183–191)}$], which fits even better to the known PACS-1 interaction motifs in terms of the number of negative charges and possible phosphorylation sites.

The interaction of Nef with the human thioesterase is the latest reported to influence Nef-mediated endocytosis (Cohen *et al.*, 2000; Liu *et al.*, 2000). This interaction has been mapped to a highly conserved and exposed cluster [$FPD_{(121)}$] in the loop connecting α4 and β2 (Figure 2), and the downregulation of both CD4 and MHC I, as well as the enhancement of infectivity, are affected by mutation of these residues. All of these observations have been linked to the loss of ability of Nef to form dimers, suggesting that the oligomerization state of Nef may be critical for its function (Liu *et al.*, 2000).

Conclusion

One of the fascinating aspects of the structure of Nef is that so many of its functions are mediated by its flexible regions. These include a membrane anchoring region of variable length, one loop within the highly ordered core domain designated for signalling and retention and a second very flexible loop for trafficking and internalization. However, it is not surprising that the flexible regions account for so many effects. They form large surfaces of the protein that are readily accessible for interactions, and they are capable of undergoing important conformational changes, e.g. for transient binding interactions. The less structured nature of these regions also correlates with a higher degree of sequence variability, a feature that enables the protein to escape the immune response. In summary, the structure of the accessory Nef protein seems not to be designed to exert any one particular function as efficiently as possible, but rather to mediate a variety of effects 'quite well'.

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