A Structure-function Analysis of the Nef Protein of Primate Lentiviruses

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Nef is one of the five so-called auxiliary genes of primate lentiviruses, a group that also includes *vif*, *vpr*, *vpu* (in HIV-1 and SIVCPZ), and *vpx* (in HIV-2 and other SIV strains). Inactivating these genes does not completely prevent viral replication in cell culture (reviewed in (Cullen, 1998)), but studies conducted both in SIV-infected monkeys and in HIV-infected individuals have revealed that they encode important virulence factors. Three major functions of Nef have been uncovered through *in vitro* analyses: the downregulation of CD4 and MHC class I, the stimulation of virion infectivity, and the capacity to alter the activation state of cells.

Nef, an important virulence factor

Nef is abundantly produced during the early phase of viral gene expression, when its mRNA represents three quarters of the viral mRNA load of the cell (Guy *et al*., 1987; Klotman *et al*., 1991). Nef stimulates viral growth both in cell culture and *in vivo*. Studies in rhesus monkeys indicated early on that Nef is important for achieving high levels of viremia and for disease induction (Kestler *et al*., 1991). Attenuated forms of HIV-1 and HIV-2 deleted in *nef* were also identified in individuals presenting as long term-non progressors (Deacon *et al*., 1995; Kirchhoff *et al*., 1995; Learmont *et al*., 1999; Mariani *et al*., 1996; Salvi *et al*., 1998; Switzer *et al*., 1998). Finally, in a recently described transgenic mouse model, Nef expression in CD4 positive cells caused an AIDS-like disease in the absence of viral replication (Hanna *et al*., 1998).

A difficult, yet rewarding, task has been to identify the molecular mechanisms hidden behind the three major known functions of Nef. A considerable amount of information has been obtained thanks to the combined power of structural, functional and genetic analyses.

General biochemical and structural features of Nef

HIV-1 Nef is a myristoylated protein of 206 amino acids that associates with cellular membranes (Niederman *et al*., 1993). The Nef proteins of HIV-2 and SIV are slightly longer, approximately 250 amino acids in length. Nef is also phosphorylated on serine and threonine, although the role of this modification is unclear (Yang and Gabuzda, 1999).

The understanding of Nef mechanisms of action has been facilitated by the determination of its three-dimensional structure. The crystal structure of the core of HIV-1 Nef complexed with the third Src homology (SH3) domain of the Fyn or Hck tyrosine kinases has been solved (Arold *et al*., 1997; Lee *et al*., 1996). Additionally, the solution structure of Nef either alone or bound to a peptide from the CD4 cytoplasmic tail has been determined by nuclear magnetic resonance (NMR) (Grzesiek *et al*., 1996a; Grzesiek *et al*., 1996b). These studies show that the core of HIV-1 Nef consists of a type II polyproline helix (aa70–77) which represents the main binding site for the Src family kinases. This domain is followed by two alpha helices (aa 81–120), a four-stranded anti-parallel beta-sheet (aa 121–186), and two additional alpha helices (aa 187–203). Residues 60–71 and 149–180 form flexible solvent-exposed loops. The three proximal helices of the Nef core domain (aa 70–120) form a cavity theoretically accessible to drugs, and compounds binding this crevice would probably disrupt interactions between Nef and Src family kinases.

The NMR structure of a peptide corresponding to the N-terminal domain of Nef has been recently obtained. This region forms a well-ordered alpha-helix from residues 6 to 22, with the N- and C-terminal regions having a less ordered structure (Barnham *et al*., 1997).

CD4 and MHC class I downregulation: Variations on the same theme?

CD4 downregulation is probably the best explained function of Nef (Aiken et al., 1994; Garcia and Miller, 1991; Guy *et al*., 1987; Mariani and Skowronski, 1993; Piguet *et al*., 1999; Rhee and Marsh, 1994). Nef alters the routing of CD4 at three levels. First, the viral protein redirects some CD4 from the trans-Golgi network (TGN) to the endosomal compartment. Second, it triggers the accelerated internalization of molecules that have reached the cell surface. And third, it targets CD4 from the endosome to the lysosome (Aiken *et al*., 1994; Kim *et al*., 1999; Lu *et al*., 1998; Mangasarian *et al*., 1997; Piguet *et al*., 1998; Piguet *et al*., 1999; Rhee and Marsh, 1994).

Nef accomplishes these effects apparently by acting as a connector between the receptor and components of the cell protein trafficking machinery. On the one hand, Nef binds to CD4, recognizing a dileucine-based signal in the membrane proximal region of the receptor cytoplasmic tail. For HIV-1 Nef, the CD4 binding site is centered around residues 57–59, and extends over the proximal region of the Nef core (Aiken *et al*., 1994; Greenway *et al*., 1995; Grzesiek *et al*., 1996; Harris and Neil, 1994; Hua and Cullen, 1997; Mangasarian *et al*., 1999; Rossi *et al*., 1996). On the other hand, Nef recruits downstream cellular partners. Of note however, it has so far not been possible to co-immunoprecipitate a complex containing CD4, Nef and one of its downstream cellular partners.

The first downstream partner of Nef is the adaptor protein complex of clathrin coated pits (CCP), or AP. Adaptor complexes are heterotetrameric structures that normally recruit clathrin to the cytoplasmic tail of receptors containing endocytosis signals. An interaction between Nef and the medium chain of adaptor complexes was demonstrated both in the yeast-two-hybrid system and *in vitro* with recombinant proteins (Le Gall *et al*., 1998; Piguet *et al*., 1998). For HIV-2 Nef and SIV Nef, this interaction required two adjacent tyrosine-based motifs in their N-terminus (Lock *et al*., 1999; Piguet *et al*., 1998) and possibly a C-terminal leucine-methione dipeptide (Bresnahan *et al*., 1999). In contrast, HIV-1 Nef interacts with AP via a dileucine-based motif in the C-terminal region of the viral protein (Bresnahan *et al*., 1998; Craig *et al*., 1998) and seems also to recruit the β chain of adaptor complexes (Greenberg *et al*., 1998). This is an interesting example of converging evolution, with HIV-1 and SIV Nef recruiting the same cellular protein complex via partly distinct determinants. Nef also interacts with a subunit of the V-ATPase that could facilitate its association with the endocytic apparatus (Lu et al., 1998). Nef mutants defective for AP recruitment are unable to accelerate CD4 internalization, either in trans or when fused to the extracellular and transmembrane domains of the receptor (Bresnahan *et al*., 1998; Craig *et al*., 1998; Greenberg *et al*., 1998; Piguet *et al*., 1998). Together with the biochemical evidence, these functional data demonstrate that adaptor complexes are major effectors of Nef-induced CD4 downregulation.

The second downstream cellular partner of Nef in this process is the COP I coatomer, another macromolocular structure involved in protein sorting. The COPI coatomer has a well known role in the ER-Golgi transport, but is also present in early endosomes (Daro *et al*., 1997; Duden *et al*., 1991; Gu *et al*., 1997; Orci *et al*., 1993; Whitney *et al*., 1995).

Nef interacts with the β subunit of COP I, both *in vitro* and in the yeast two-hybrid system (Benichou *et al*., 1994). Mutating a diacidic (EE^{155})-based motif in HIV-1 Nef abrogates the ability of a GST-Nef fusion protein to recruit β-COP from cytoplasmic extracts, and prevents Nef-induced CD4 lysosomal targeting (Piguet et al., 1999). Furthermore, in a CHO cell line derivative containing a temperature sensitive mutant of one subunit of the COP I coatomer (Guo *et al*., 1996), Nef is unable to direct CD4 to lysosomes. These results suggest not only an explanation for this step of transport but also reveal Nef as the first demonstrated endosomal cargo for the COP I coatomer (Piguet *et al*., 1999). However, β-COP does not seem the sole Nef partner implicated in this process. Indeed, additional cellular proteins strongly regulate the interaction of Nef with the COP I coatomer (Piguet et al., 1999).

In summary, a combination of functional, biochemical and structural analyses point to a model in which HIV-1 Nef contains at least four distinct determinants crucial for efficient CD4 downregulation,

each carrying out one step of this process. The N-terminal myristoylation signal directs Nef to membranes, its primary site of action. A second domain, centered around amino acids 57 to 59, binds the CD4 cytoplasmic tail. A third motif, which includes the LL^{165} dipeptide, is responsible for recruiting adaptor complexes, thereby triggering the formation of CD4-specific CCP which rapidly internalize the receptor. The nearby DD^{174} dipeptide binds to a subunit of the V-ATPase that might facilitate AP-2 recruitment. Finally, the EE^{155} diacidic sequence mediates the binding of Nef to COP I in endosomes, and as a result governs the lysosomal targeting and degradation of Nef-associated CD4 molecules. More schematically, the CD4 binding domain is concentrated in the N-terminal flexible loop and the core domain of Nef, whereas the recruitment of the downstream partners AP and COP I is mediated by a C-terminal flexible loop.

Recently, some light has been shed on the role of CD4 downregulation in the HIV replicative cycle. Indeed, high levels of CD4 on the surface of HIV-producing cells inhibit the infectivity of released virions by trapping the viral envelope. Nef, and to a lesser extent Vpu, another CD4-downregulating protein of HIV-1, counteract this negative effect of CD4 (Lama *et al*., 1999). Very high CD4 levels might even block virion release from HIV-1 infected cells (Ross *et al*., 1999). Other benefits of Nef-induced CD4 downregulation could stem from the liberation of Lck that accompanies the binding of Nef to the CD4 cytoplasmic tail. Increased levels of free Lck could promote T cell activation, thereby creating a milieu favorable for viral gene expression. Finally, CD4 downregulation could prevent potentially lethal superinfection events. Of note, these effects are not mutually exclusive, and together might explain why Nef-induced CD4 downregulation is so well conserved amongst primate lentiviruses.

MHC class I downregulation: mysterious paths

Nef induced-MHC class I downregulation is less efficient than the modulation of CD4, and its mechanism is less well understood. A tyrosine found in the cytoplasmic tails of HLA-A and B, but not of HLA-C, is critical for response to Nef (Le Gall et al., 1998). In the presence of the viral protein, MHC class I is internalized more rapidly, routed to the trans Golgi network, and ultimately degraded (Greenberg et al., 1998; Le Gall *et al*., 1998; Schwartz *et al*., 1996). Residues in Nef essential for MHC class I downmodulation are distinct from those necessary for CD4 downregulation (Greenberg *et al*., 1998; Mangasarian *et al*., 1999). Regions of Nef implicated in alterating intracellular signalling pathways, such as the N-terminal alpha-helix, the proximal acidic cluster and the proline-based repeat, are also important for Nef-induced MHC class I regulation. In spite of this, MHC downregulation by Nef does not seem to involve the phosphorylation of the receptor and is not blocked by tyrosine kinase inhibitors. The AP recruiting motif of Nef (LL^{165}), essential for regulating CD4, is completely dispensable for MHC class I modulation (Greenberg *et al*., 1998; Mangasarian *et al*., 1999). This data suggests that Nef affects CD4 and MHC class I with the help of different cellular partners, which remain to be identified.

While the mechanism of Nef-induced MHC class I downmodulation is still uncertain, its biological significance is better understood. Indeed, MHC I presents antigenic peptides on the cell surface and permits the recognition and killing of cells expressing foreign proteins by cytotoxic T lymphocytes (CTL). Nef allows HIV-infected cells to evade CTL, at least to some extent (Collins *et al*., 1998). Of note, Nef is unable to induce HLA-C downregulation. This allows infected cells to escape natural killer (NK) cells that would attack a cell completely devoid of surface MHC class I (Cohen *et al*., 1999). In vivo, a strong CTL response is observed against viral epitopes (including Nef). Nevertheless, HIV infection cannot be controlled by the immune system, suggesting that immune evasion is crucial in the course of the disease (reviewed in (McMichael, 1998)).

Nef, kinases and signaling alterations

Nef can recruit several protein kinases involved in cell signaling. For example, Nef interacts directly with the SH3 domain of the Hck and Lyn non-receptor protein tyrosine kinases via a conserved PxxP motif in its core domain, as demonstrated by both structural and biochemical data (Lee *et al*., 1996; Saksela *et al*., 1995). Another partner of Nef is the T cell-specific Lck tyrosine kinase, apparently bound via both the N-terminus and the central region of the viral protein (Baur et al., 1997). Nef

also interacts with a member of the p-21 activated kinase (PAK) family (Sawai *et al*., 1996) as well as with another yet unidentified serine/threonine kinase, and with the theta subunit of protein kinase C (Smith *et al*., 1996);. Finally Nef can recruit Vav, a guanine nucleotide exchange factor (GEF) for the Rho-family GTpases, that in turn activates PAK and additional downstream effectors of Nef including cdc42 and Rac1(Fackler *et al*., 1999). Several additional signaling-involved molecules associate with HIV-1 Nef, including Raf1 and the TCRζ (Hodge *et al*., 1998; Xu *et al*., 1999). Nef binding to TCRζ seems to induce an up-regulation of FAS ligand, which could further contribute to HIV immune evasion by killing cytotoxic T cells that come into contact with virus infected cells (Xu et al., 1997).

These multiple interactions place Nef at the heart of cellular activation pathways. Several recent studies point towards a model in which Nef sensitizes T cells to activation via the TCR. In transgenic mice, expression of the full HIV-1 viral genome under the control of the CD4 promoter triggered a disease reminiscent of AIDS (Hanna *et al*., 1998). The major determinant of pathogenicity was Nef, which induced the activation and hyperresponsiveness of thymocytes to anti-CD3 antibody stimulation, thereby promoting the tyrosine phosphorylation of several substrates, including LAT and MAPK (Hanna *et al*., 1998). Another hint for the Nef-induced activation of T cells came from particular SIV Nef variants that harbor amino acid sequences resembling immunoreceptor tyrosine-based activation motifs. Viruses carrying these *nef* alleles could replicate to high levels in peripheral blood mononuclear cells without a need for exogenous stimulation, and could induce IL-2 production (Du *et al*., 1995). Similarly in human primary T lymphocytes HIV-1 Nef can induce a state of TCR hyperresponsiveness (Schrager and Marsh, 1999; Wang *et al*., 2000). Finally, in Jurkat human T lymphoid cells, the surface expression of a CD8-Nef chimera resulted in the appearance of T cell activation markers, in the accumulation of tyrosine phosphorylated proteins, in the induction of NF-kB activity and, ultimately, in cell death by apoptosis. In contrast, the intracytoplasmic accumulation of the CD8-Nef chimera was accompanied by inhibition of TCR signaling (Baur *et al*., 1994). This points to a spatial regulation of Nef activity. In that respect, Nef was recently found to associate with membrane microdomains known as rafts (Wang *et al*., 2000). Rafts represent discrete subdomains of the plasma membrane that concentrate glycophosphatidylinositol-linked proteins, glycosphingolipids, and mediators of T cell activation, including Lck, Fyn and LAT. The presence of Nef in rafts is therefore most likely central to its ability to prime the T cell for activation.

In monkeys, an SIV strain encoding a Nef protein that contained an SH2 binding domain in its N-terminus induced lymphocyte activation in tissue culture and a rapidly progressive disease in monkeys (Du *et al*., 1995). SIV mutants containing alterations in a central di-arginine motif involved in PAK recruitment initially generated low levels of viremia, but quickly reverted to a wild-type genotype and to full virulence (Sawai *et al*., 1996). Of note, mutations in these residues also affect Nef-induced CD4 downregulation, complicating the interpretation of this result. SIV strains with mutations in the proline rich domain important for interaction with Fyn and Hck induced opposite phenotypes in two distinct studies (Khan *et al*., 1998; Lang et al., 1997). However the general trend in both animal models and *in vitro* experiments support a model where Nef sensitizes T cells to an activation via their TCR, rendering them more susceptible to efficient viral replication.

Additionally, Nef is also able to promote the production, by HIV-1-infected macrophages, of macrophage inflammatory proteins (MIP) 1α and 1β, and of an as yet unidentified mediator of T cell activation. These chemokines then induce the chemotaxis and activation of nearby resting T lymphocytes, facilitating their subsequent infection (Swingler *et al*., 1999).

Nef boosting virion infectivity

In single-round infectivity assays, Nef-defective viruses produced from CD4-negative cells are from three to ten times less infectious than wild type particles. Additionally, Nef-mutated virions show a severe growth defect in primary blood lymphocytes infected while resting and subsequently activated (Miller *et al*., 1994; Spina *et al*., 1994). Nef can thus enhance the infectivity of HIV-1 virions. At least two distinct mechanisms seem involved. First, Nef stimulates HIV-1 infectivity in a CD4-independent manner. Second, as described above, Nef-induced CD4 downregulation counterbalances the inhibitory effect that high levels of cell surface CD4 can exert on virion release and envelope incorporation (Lama *et al*., 1999; Ross *et al*., 1999).

The CD4-independent stimulation of virion infectivity by Nef translates in a more efficient synthesis of proviral DNA after virions penetrate target cells (Aiken and Trono, 1995; Schwartz *et al*., 1995). However, the Nef requirement is conditioned by the route of viral entry. Nef is thus completely dispensable if HIV-1 particles are pseudotyped with the G protein of vesicular stomatitis virus, that is, are internalized via receptor-mediated endocytosis rather than by fusion at the plasma membrane (Aiken, 1997).

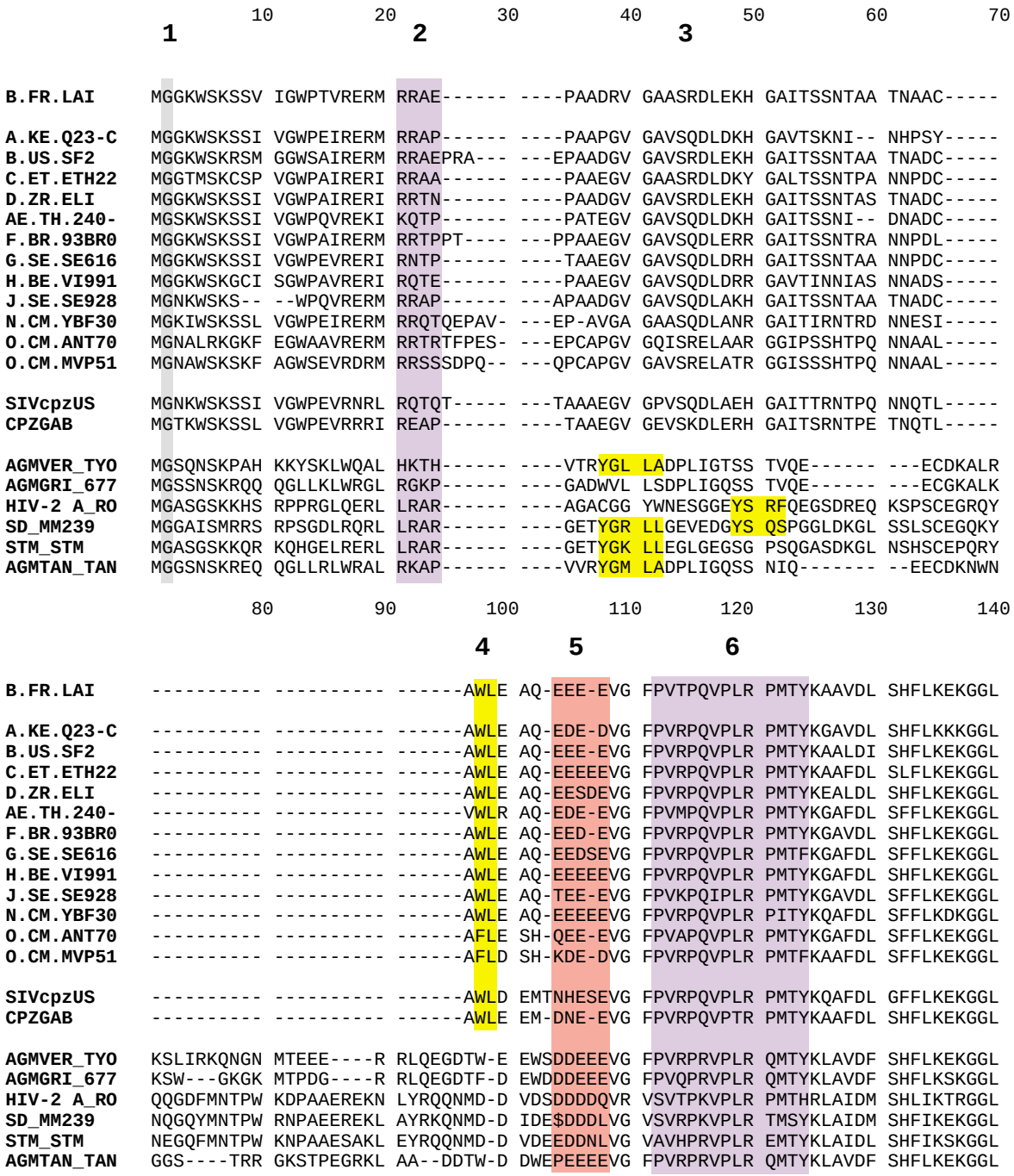
The biochemical events that underlie Nef-induced stimulation of HIV-1 infectivity are still obscure. Viruses expressing HIV-1 Nef variants mutated in the LL^{165} dileucine motif are impaired for both infectivity and Nef-mediated CD4 downregulation (Craig *et al*., 1998). Residues necessary for association with cellular protein kinases are also important for this effect of the viral protein: a proximal sequence extending between amino acids 17 and 26, and the proline-rich region in the central core of the viral protein (Baur *et al*., 1997; Saksela *et al*., 1995). Furthermore, an arginine dipeptide involved in recruiting PAK contributes to both CD4-dependent and CD4-independent enhancement of virion infectivity. Interestingly, an HIV-1 strain mutated in this di-arginine motif and unable to recruit PAK had lower levels of serine phosphorylation of matrix, one of the main structural components of the virion (Swingler *et al*., 1997). There are also indications that a cellular kinase of the MAPK family is incorporated into virions (Jacque *et al*., 1998). This kinase can induce the phosphorylation of the viral matrix protein, which in turn might increase viral infectivity at a point preceding the transcriptional activation of the provirus. Moreover, virus infectivity is enhanced by treatment of cells with MAPK stimulators, such as phorbol myristate acetate, as well as by coexpression of constitutively active MEK (MAPK kinase). Treatment of cells with a specific inhibitor of MAPK activation, or with a MAPK antisense oligonucleotide reduces the infectivity of HIV-1 virions without significantly affecting virus production (Yang and Gabuzda, 1999).

Between 60 and 200 molecules of HIV-1 Nef are incorporated in virions, where most are cleaved by the viral protease between residues 57 and 58 and associate with the viral core (Kotov *et al*., 1999). The functional significance of Nef cleavage is unclear, because a proteolysis-resistant mutant (WL^{58} AA) is still able to enhance infectivity. Moreover SIV Nef, which is not cleaved by the HIV-1 protease, can also stimulate virion infectivity (Chen *et al*., 1998; Freund et al., 1994; Pandori *et al*., 1996; Welker *et al*., 1996). The exact roles of the virion incorporation of Nef and of its cleavage by the viral protease thus need further clarification.

Conclusion

Nef, as an important factor in the pathogenesis of AIDS, is an attractive target not only for basic investigation, but also for drug discovery (reviewed in (Miller and Sarver, 1995)). The structural data offer some clues as to how inhibitory molecules could be developed. A compound that blocks the PxxP domain of Nef would be expected to have a dually beneficial effect. Not only would viral replication be slowed down, but infected cells would be more efficiently recognized by the immune response. The structural analyses reveal that the PxxP-based pocket of Nef is distinct from related cellular determinants involved in recognizing SH3-containing proteins, as these motifs are usually constituted of continuous amino acids (Grzesiek *et al*., 1996). This unique feature of Nef could allow the development of drugs that would inhibit the viral protein while sparing its cellular homologues.

In a more fundamental perspective, Nef is at the interface between HIV and its host cell. Investigating the mechanisms of Nef action therefore provides important information not only on the virus itself, but also on pathways that govern cellular events such as protein trafficking and signal transduction. The study of Nef can thus help to elucidate biological processes that are keys to the homeostasis and survival of the cell.



Sequence alignment of Nef proteins from HIV-1, HIV-2 and SIV isolates, in single-letter amino acid code, with conserved residues identified as playing a role in specific Nef functions indicated with colors

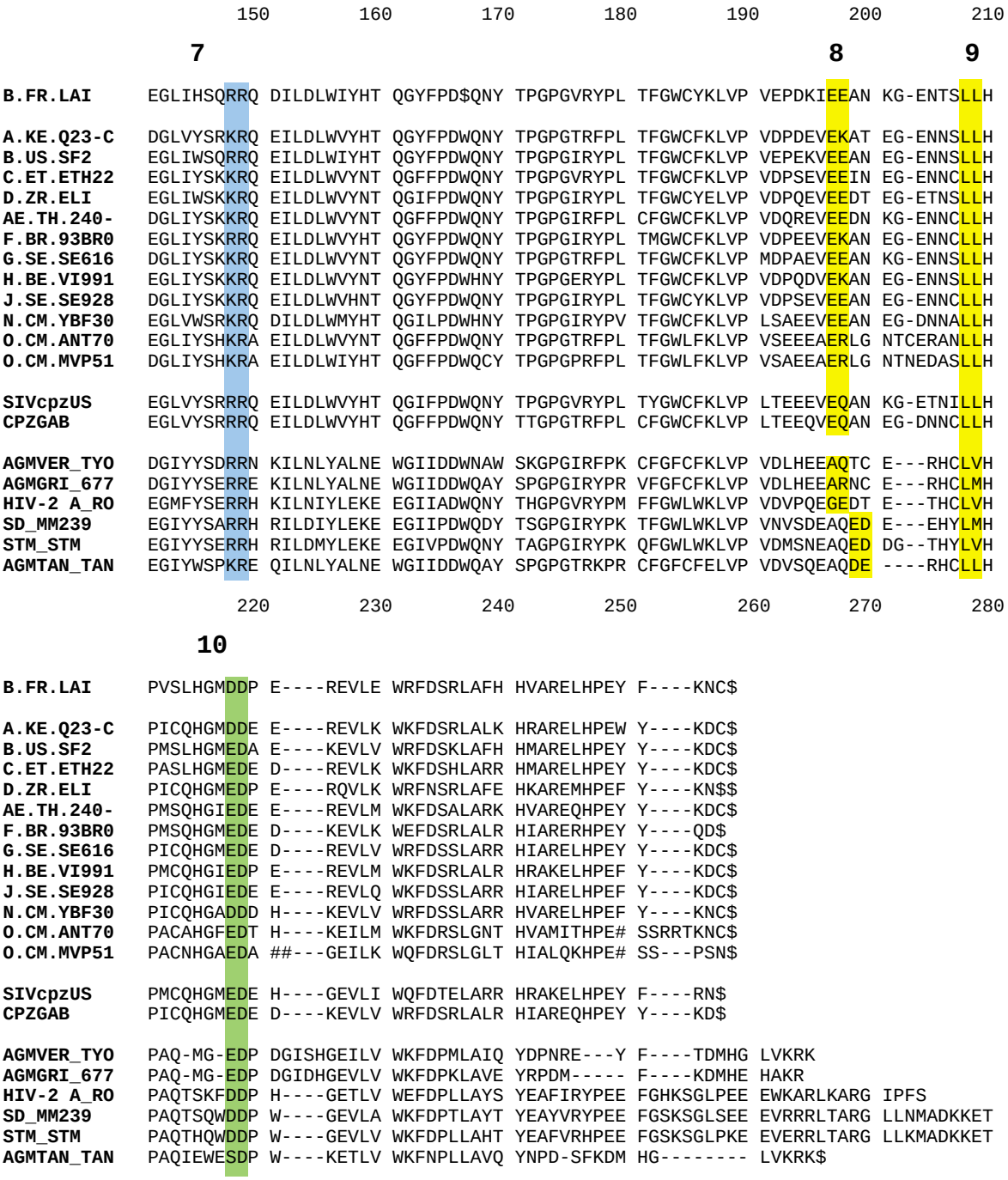
CD4 downregulation

MHC I downmodulation + association with signaling molecules

MHC I downmodulation

CD4 downregulation + MHC I downmodulation + association with signaling molecules

Bold numbers on top refer to the following: **1**: myristoylation site. **2**: N-terminal α-helix (MHC I downregulation + protein kinase recruitment). **3**: tyrosine-based AP recruitment (HIV-2/SIV Nef). **4**: heart of CD4 binding site (characterized only for HIV-1 Nef). **5**: acidic cluster (MHC-I downregulation).**6**: proline-based repeat (MHC-I downregulation + SH3 binding).



Sequence alignment of Nef proteins continued. Conserved residues identified as playing a role in specific Nef functions indicated with colors

CD4 downregulation

CD4 downregulation + association with signaling molecules

association with signaling molecules

Bold numbers on top refer to the following: **7**: PAK binding. **8**: COP I recruitment. **9**: di-leucine based AP recruitment (HIV-1 Nef). **10**: V-ATPase and Raf-1 binding.

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