[Frontiers in Bioscience 5, d268-283, February 1, 2000]

INTERACTIONS OF HIV-1 NEF WITH CELLULAR SIGNAL TRANSDUCING PROTEINS

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1. ABSTRACT

Nef is a 27 - 34 kD myristoylated protein unique to primate lentiviruses. A functional Nef gene is important for development of high viremia and simian AIDS in SIV infected rhesus macaques (1). In a transgenic mouse model expression of Nef protein alone when expressed under a CD4-promoter is sufficient to cause an AIDS like disease (2). A critical role for Nef in development of AIDS in humans is suggested by the observation that some individuals with a long-term nonprogressive HIV-1 infection are infected with viruses carrying naturally occurring Nef deletions (3-5). The mechanism of Nef action remains incompletely understood, but multiple lines of evidence point out to a role in modulation of cellular signaling pathways via physical and functional interactions with host cell proteins.

2. PHENOTYPIC EFFECTS OF Nef EXPRESSION IN CELL CULTURE

Although the role of Nef *in vivo* during the development of AIDS has been clearly demonstrated, the underlying cellular and molecular mechanisms are less clear. Replication of HIV in most transformed cell lines does not depend on a functional Nef gene. A positive effect of Nef on HIV replication kinetics can, however, be demonstrated under certain cell culture conditions, such as infection of resting peripheral blood mononuclear cells (PBMCs) which are subsequently incubated for a couple of days before stimulation of the culture to allow the virus to spread (6,7). The number of different phenotypic effects caused by Nef expression *in vitro* suggests that Nef has multiple functions that contribute to its pathogenic function in infected individuals, and that it may be important in different steps of HIV life cycle. A list of possible and partially overlapping mechanisms that could explain Nef-induced HIV-1 disease progression is presented in Table 1. The significance and relative importance of these possible *in vivo* mechanisms are currently not known, as this list is mainly based on extrapolation from *in vitro* work on the Nef functions discussed in the following.

2.1. Downregulation of CD4 and MHC I molecules

The best-known phenotypic effect of Nef *in vitro* is the downregulation of the cell surface expression of the CD4 receptor for the virus by a posttranslational mechanism (8-12). Also the cell surface expression of major histocompatibility complex class I (MHC I) molecules becomes downregulated (13,14). A number of other proteins whose cell surface expression is also regulated by endocytosis are not affected by Nef, indicating that these effects are relatively specific (14,15).

Normally, CD4 downregulation occurs upon serine phosphorylation-triggered release of the Src family

Table 1. Possible pathogenic mechanisms of Nef *in vivo*

- Increased infectivity of viral particles.
- Enhanced viral gene expression.
- Facilitation of the budding of viral progeny.
- Prevention of cells from superinfection.
- Modulation of apoptosis.
- Evasion from CTL response.
- Deregulation of cytokine networks of the immune system.
- Increased number of new target cells by activation of bystander cells.

protein-tyrosine kinase $p56^{lck}$, which is bound to two cysteine residues in the cytoplasmic domain of CD4. The dissociation of the CD4-$p56^{lck}$ complex results in the exposure of a neighboring dileucine motif, which is a recognition signal for the endocytosis apparatus that transports CD4 to the lysosomal compartment (16). Nef was found to physically bind to this same dileucine motif in the cytoplasmic tail of CD4 (15,17-19). This interaction induces CD4 downregulation in a manner independent of serine phosphorylation (9), both in Lck-positive and Lck-negative cells (20). A conserved dileucine motif in HIV-1 Nef itself acts as a lysosomal targeting signal, and has been found to interact with components of the endocytic machinery (21-29). Also other regions in Nef are found to be important for the binding with CD4 and for the dissociation of Lck from CD4 (19,30-33).

It has been speculated that the biological significance of downregulation of CD4 might be to enhance HIV replication by preventing a potentially hazardous superinfection of cells (34). Furthermore, high levels of CD4 might interfere with production and release of virions from the cell surface (35) (see below). On the other hand, the downregulation of CD4 could be a by-product of some other more consequential event, for example liberation into the cytoplasm of Lck that is normally bound to the intracellular tail of CD4 and has a role in the activation of T-cells (16,31,36-38).

Downregulation of MHC I molecules, which do not contain a dileucine motif, also results in their accumulation in the lysosomes (14). The determinants in Nef, namely the N-terminal alpha helix and the proline rich region, are distinct from those required for the downregulation of CD4, pointing to a different mechanism (39,40). In the case of MHC I, suggested pathophysiological roles for its downmodulation by Nef involve decreasing the amount of MHC I molecules that get incorporated into the envelopes of virus progeny, and in particular, escape of HIV-infected cells from recognition and elimination by cytotoxic T cells (13,41,42).

2.2. Nef increases the infectivity of virus particles

Although Nef constitutes the majority of all viral proteins synthesized during the early post-integration stage of HIV infection, suggesting a principal role for the protein during this stage, several findings point to a role for Nef during the very last steps of HIV life cycle. The observed lower infectivity of HIV particles that carry a deleted Nef gene can be complemented by expression of Nef from a separate vector during virus production, but not by ectopic Nef expression in the target cells of infection (6,43-47). This producer cell-derived effect then somehow facilitates a step that occurs after viral entry but before *de novo* synthesis of viral proteins (including Nef) in the target cell, and is manifested by an increased efficiency of reverse transcription of the viral genome (43,45,47).

There are several potential mechanisms by which the expression of Nef during virus production could lead to more infectious particles. These include effects of Nef caused by its presence in the virion, effects triggered by the Nef molecules that would get delivered into the target cell, or effects by Nef in the producer cell that would influence virion composition. An average of 10-100 Nef molecules per virion have been shown to be present in HIV particles (48-51). Most of these incorporated Nef molecules are cleaved by the HIV protease in a highly conserved region between the membrane-anchoring aminoterminus and the conserved Nef core domain (48,50). Although such cleavage would seem to inactivate Nef, it could also be hypothesized that the resulting proteolytic products might serve a specific function. While mutations in Nef that prevent its cleavage by the HIV protease are indeed deficient in their ability to increase HIV particle infectivity, it has been found that also other mutations in this conserved region of Nef that do not affect protease cleavage also give a similar phenotype (52-54), thus leaving the significance of proteolytic cleavage of Nef unresolved.

Alternatively, Nef might not have a specific role in HIV particles *per se*, and its incorporation into these could rather serve to recruit Nef-binding host cell proteins into the virions. To this end, it has been shown that expression of Nef increases the incorporation of a cellular serine kinase into the virions, which may cause the observed Nef-induced increase in viral matrix phosphorylation (55), which in turn may be associated with increased infectivity of the particles (56). Finally, packaging of Nef in the virions might be altogether irrelevant and unrelated to the increased infectivity of Nef(+) viruses. In this scenario effects of Nef on the intracellular environment of the producer cell would result in an increased infectious capacity of the progeny viral particles, e.g. via altered posttranslational modification of viral components during HIV particle assembly.

Several studies have shown that downregulation of CD4 does not have a critical role in the increased infectivity of virus particles produced in Nef-expressing cells. HIV particles produced in cells lacking CD4 also display such Nef-induced increase in infectivity (6,13). Moreover, we have shown (57), and a number of studies have subsequently confirmed (30,33,58,59), that CD4 downregulation by Nef can be genetically separated by site-directed mutagenesis from its ability to enhance HIV replication in PBMCs and to increase HIV particle infectivity, in particular by changes involving amino acids forming a conserved proline-repeat (PxxP) motif of Nef (see below).

Nevertheless, recent studies have shown that the downregulation of CD4 may have an additional positive effect on HIV infectivity, which would operate at the level of virus entry. Using cells that express high levels of CD4, Lama *et al.* (60) have shown that the failure of Nef(-) viruses to downregulate CD4 results in less infectious virions due to decreased incorporation of Env. In contrast, however, Ross *et al.* (61) who used essentially the same experimental system, reported in an accompanying paper that Nef(+) and Nef(-) viruses produced in CD4-overexpressing cells are equally infective, but Nef(-) viruses are being produced in lower amounts due to their inhibited shedding. In the light of these differing results and the earlier negative results from studies using cells with more physiological levels of CD4 (45), a role of Nef-induced CD4 downregulation in regulating HIV particle infectivity thus remains uncertain.

2.3. Nef affects host cell signal transduction pathways

Besides acting late in the replicative cycle to increase HIV particle infectivity, Nef expression is thought to contribute to optimal viral replication and AIDS-associated pathogenesis in a number of other ways. The multiple interactions revealed between Nef and cellular signal transducing proteins (discussed below) have suggested that many of these Nef functions would be achieved by altering gene expression in the host cells.

A large and growing number of effects by Nef on the activity of various components of cellular signaling cascades, such as second messengers and transcription factors, have often been reported (30,62-88). It is, however, not easy to build a coherent picture from these observations. One apparent problem is that opposing effects have been reported regarding the same signaling events, such as those involved in the response initiated by triggering of the T cell receptor and leading to transcriptional activation of the IL-2 gene and the HIV provirus (see references above). In addition to the molecular clone of Nef used in these studies, such differences might be due to a number of variables, including the selection of the cell line and culture conditions, and the level and duration of Nef expression. As a consequence, the relevant cellular effects of Nef might become obscured by inappropriate subcellular distribution of Nef, titration out of critical cellular partners of Nef, depletion of relevant second messenger molecules, or other such reasons that could be categorized as "toxicity" or "squelching".

Although inhibition of certain cellular signaling pathways may be a pathophysiologically relevant function of Nef, because of the positive correlation between HIV replication and cellular activation, it would seem logical to assume that in most cases the effects of Nef on host cell signaling would be positive. Experimental evidence of such positive signaling effects by Nef has indeed been reported during the past couple of years.

The most extreme example of signaling abnormalities induced by Nef is its ability to promote malignant transformation. An unusually potent allele of SIV Nef has been shown to induce morphological transformation of immortalized 3T3 fibroblasts (63). Also a native HIV Nef allele was also shown to cooperate with a cellular proto-oncogene product, protein tyrosine kinase Hck, in transforming another immortalized fibroblast cell line, Rat2 (89). This involves deregulation by Nef of the normally tightly controlled kinase activity of Hck (89), which in turn leads to a number of downstream effects, such as activation of the AP-1 transcription factor (78).

While such changes in host cell transcription might directly increase the expression of the HIV genome itself, they could also modify the internal and external environment of the infected cells in ways that would indirectly increase their ability to support HIV replication, promote their survival, facilitate spreading of the virus to neighboring cells, or interfere with antiviral immunity. An intriguing example of a disease-promoting paracrine effect of Nef was recently reported by Swingler and colleagues (90). They showed that expression of Nef in macrophages caused these cells to produce the T lymphocyte chemotactic CC-chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β, as well as an as yet unidentified T cell activating protein (90), which together allowed neighboring resting T cells to become susceptible for HIV infection.

On the other hand, an example of autocrine stimulation of T cells induced by Nef has been provided by Desrosiers and colleagues (91), who observed that the significant growth advantage of Nef(+) SIV strains in a Herpes saimiri-immortalized IL-2-dependent T cell clone was caused by endogenous IL-2 production induced by Nef in these cultures. It remains to be examined if a similar auto/paracrine effect also explains the enhanced replicative kinetics observed for Nef(+) viruses (HIV and SIV) in suboptimally stimulated primary cell cultures, a Nef function that appears to be distinct from its ability to increase HIV particle infectivity (92).

Finally, modification of host cell signaling cascades may underlie Nef-mediated evasion of HIV-infected cells from immune clearance. The requirement of SH3-binding capacity of Nef for induction of downregulation of HLA-I suggest that this event might be somehow triggered via an effect of cellular signaling networks (39,40). Nef-induced upregulation of FasL, the proapoptotic ligand for Fas (Apo-1, CD95) receptor is another reported mechanism by which Nef may protect infected cells from cytotoxic T cell attack and also lead to destruction of such HIV-specific CTLs (93-97). Fas ligand expression is regulated by a number of transcription factors, such as NFAT, NF-κB and AP-1 (98 and references therein), some of which have been found to be regulated by the expression of Nef as discussed above. In addition, some data suggest that Nef-induced deregulation of host cell cytokine gene expression may interfere with a proper antiviral immune response via more global effects caused by an imbalance of cytokine networks of the immune system (70).

3. CELLULAR PARTNERS AND TARGETS OF Nef

A key to understanding how Nef mediates its intracellular functions will be the identification of the

Table 2. Cellular proteins reported to bind HIV-1 or SIV Nef

Signaling proteins
• *Src family tyrosine kinases*
• *Hck (57,79,89,109,110,114, 121-123)*
• *Lyn (57, 122)*
• *Fyn (109,111, 122,124)*
• *Lck (72,81,82,121-123)*
• *Src (63, 151*
• *PAK2 (59,73,133-139)*
• *Protein kinase C theta (145)*
• *Mitogen-activated protein kinase Erk-1 (72,82)*
• *Raf1 (146)*
• *TCRz chain (95,147,148)*
• *Vav1 and Vav2 (86)*
Proteins implicated in CD4 downregulation
• *CD4 (17,19,72,157)*
• *p35 thioesterase (160)*
• *b-COP (161)*
• *Actin (162)*
• *Adaptins (22-24,26,163)*
• *V-ATPase (25)*

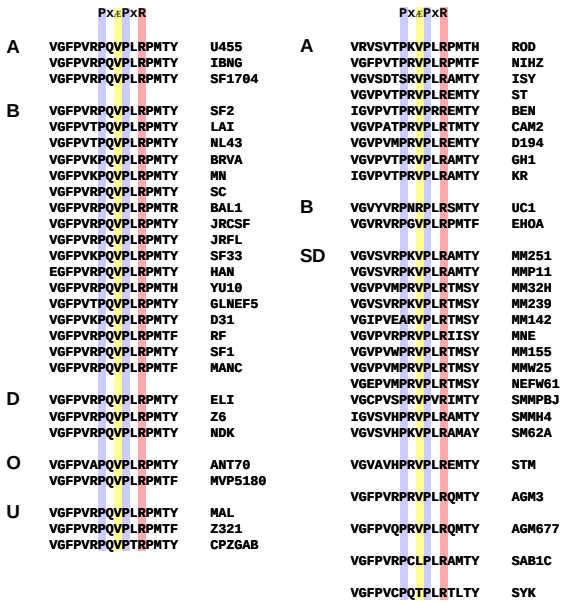

Figure 1. Evolutionary conservation of SH3 binding site in Nef sequences from different primate lentiviruses. The sequence PxØPxR (where x is any amino acid and Ø is a hydrophobic aliphatic residue) is, besides the HIV-1 Nef sequences shown (left panel), conserved in more than 95% of HIV-1-like sequences available in the Los Alamos HIV sequence database (http://hiv-web.lanl.gov/) including Nef from the O-subtype HIV-1 and chimpanzee SIV. This motif is also very well conserved among Nef sequences from HIV-2 as well as SIVs from different monkey species (right panel), although single amino acid substitutions to the perfect consensus are occasionally seen.

immediate cellular partners that it interacts with. A large number of cellular proteins have already been reported to bind to Nef (Table 2). Notably, with the exception proteins that are involved in CD4 downregulation, which will not be discussed in the following, most Nef-interacting proteins found so far are components of cellular signaling pathways.

3.1. Nef associated SH3 domain-containing proteins

3.1.1. The PxxP motif of Nef

The strong sequence conservation of the proline repeat motif, PxxP motif (where x is any amino acid), in Nef proteins of different primate lentiviruses, together with the functional data on mutations that disrupt it (particularly in the case of HIV-1 Nef; see later), suggest an important role for this motif in mediating the pathogenic functions of Nef. The PxxP motif has been identified as the minimal consensus sequence defining the ligands of SH3 (Src homology 3) domains (99,100). SH3 domains are modular protein units typically consisting of approximately 60 amino acids, and in most cases serving to mediate protein-protein interactions involving cellular signaling proteins, such the Src family of cytoplasmic protein tyrosine kinases (101-103). In addition to Src itself this kinase family consists of eight known members, Blk, Fgr, Fyn, Hck, Lck, Lyn, Yes, and Yrk (104,105). An important function of the Src kinases is to relay signals from outside of the cell that are mediated by transmembrane proteins lacking independent catalytic activity.

The structural basis of the SH3/PxxP interaction is now well understood (102-103,106-108). The PxxP containing peptides adopt a secondary structure known as the polyproline type II (PPII) helix, in which exactly three residues constitute one left-handed helical turn, thus placing adjacent to each other one side of the helix the two proline residues that typically define a PxxP motif. Additional proline residues are also often present, and may stabilize the PPII helix, while the PxxP defining prolines provide the important hydrophobic contacts with the binding surface of the SH3 domain.

Although the molecular details regarding SH3/ligand complexes were not yet elucidated at the time when HIV-1 Nef was first shown to bind to the SH3 domains of a subset of Src kinases (57), it is noteworthy to point out how well the amino acid sequences of the available large number of HIV-1, HIV-2 and different SIV Nef proteins conform to the consensus for a “minus“ orientation SH3 ligand. Virtually all HIV-1 Nef amino acid sequences and a great majority of those of HIV-2 and SIV contain this motif (Figure 1).

Mutagenesis and X-ray crystallographic studies have shown that in addition to the relatively idiotypic SH3/PxxP-interaction, the binding of HIV-1 Nef to the SH3 domain of Hck involves other tertiary interactions between these molecules (109,110). This helps to explain the unusually high affinity of the Hck/Nef interaction as compared to previous data on binding of SH3 domains to short peptide ligands, as well as its distinct specificity. In particular two highly conserved aromatic residues (Phe90 and Trp113 in HIV-1 NL4-3 Nef) on each side a hydrophobic crevice between the two antiparallel alpha helices forming part of the conserved core of Nef provide it

with specific affinity for Hck (and Lyn; see below) (110). By contrast the affinity of Nef to Fyn is only about 1% of that of the Hck/Nef interaction (109), despite the conservation between Hck and Fyn of most of the residues that accommodate and coordinate the binding to the PxxP peptide (109,111).

3.1.2. Hck and Lyn

Among the SH3 domains tested, those of the Src family tyrosine kinases Hck and Lyn have shown by far the highest affinity for binding to Nef. The affinity of Nef/Hck-SH3 interaction has a kD value of approximately 0.2 μM representing on of the tightest SH3/ligand interaction reported so far (109). Although the interaction between Lyn-SH3 and Nef has not been biochemically characterized as thoroughly as that involving Hck-SH3, semiquantitative data from *in vitro* and yeast interaction trap assays suggest that these two are of similar strength (57; M. Hiipakka and K.S., unpublished data). The similar tight binding of Hck and Lyn SH3 domains to Nef can be attributed to their homology in a region known as the RT-loop, which is otherwise poorly conserved among different SH3 domains. Notably, Lyn is the only other known SH3 domain (besides Hck), that has an isoleucine residue in its RT-loop in a position that was shown to be critical for the Hck/Nef interaction (109). Therefore, although the following discussion focuses on the possible role of Hck in the pathogenesis of AIDS, many of the same arguments also apply for the lesser studied Lyn, which, in addition to its avid binding to Nef, shares many biological properties and an overlapping expression pattern with Hck.

SH3-mediated co-immunoprecipitation with Nef of transfected (89) as well as endogenous (79) Hck has been reported. This interaction has striking functional consequences, and can even cause malignant transformation. The mechanism of co-transformation by Nef and Hck was shown to involve deregulated growth signaling caused by enzymatic activation of Hck (89).

The transforming potential of coexpressed Nef and Hck is in good agreement with earlier observations that certain mutations in the SH3 and SH2 domains of Src kinases can result in their catalytic activation and render them transforming (112,113). Indeed, it was shown using purified recombinant proteins *in vitro*, that binding of Nef to the SH3 domain of Hck is a powerful way of activating this kinase by overriding such SH3/SH2-mediated autoinhibition (114). The three-dimensional structure of an almost complete Hck protein (115), together with a similar structure of Src (116) and a structure of an active catalytic domain of Lck (117), have provided molecular basis for this phenomenon by demonstrating the role of the SH3 domain in locking the kinase domain in an inactive state which can be pushed to the catalytically active conformation by interaction with a protein like Nef.

The results from these biochemical, cellular, and functional studies have made Hck an attractive candidate for a cellular accomplice of Nef. However, the pattern of Hck expression suggests that its role may be limited to a subset of infected cells. Of the two cell lineages generally considered to be important for HIV infection, the T lymphocytes and monocyte/macrophages, only the latter expresses significant amounts of Hck (105). Since most of the HIV replication takes place in CD4-positive T lymphocytes, and their depletion is a critical process in the development of AIDS, one would have to postulate that the Nef/Hck interaction in monocyte/macrophages would indirectly facilitate HIV infection in the T cell compartment. As HIV infected monocyte/macrophages are relatively long-lived, they could be important in spreading the virus by simultaneously providing an activating signal as well as infectious virus to a large number of resting, and therefore HIV infection resistant, T cells which they meet. As already mentioned, he production of T cell-attracting chemokines (MIP-1α and MIP-1β) and stimulatory molecules by Nef expressing macrophages has recently been reported, although it has not been shown that this effect would be dependent on the Nef PxxP motif (91).

On the other hand, myeloid cells have also been directly implicated as important reservoirs for HIV replication during early as well as late stages of HIV disease. Specialized type of antigen presenting cells of the myeloid lineage known as Langerhans cells or tissue dendritic cells can be infected by HIV, and have been suggested to play an important role in the early spread of HIV infection by transporting the virus to the lymphoid compartment, and by having a capacity to fuse with T cells, and to support high level virus replication in such syncytias in the absence of exogenous cellular stimulation (118,119). Furthermore, it was recently reported that in AIDS patients with decreased CD4 T cell counts and opportunistic infections, the majority of the high viremia was produced by monocyte/macrophages (120).

Despite such considerations, it is likely that the role of Nef in modulating cellular signaling and promoting the pathogenesis of AIDS is not limited to myeloid cells. Also, the PxxP-motif of Nef has been shown to be critical for multiple effects of Nef on HIV infectivity and signal transduction in many different cell types, including T lymphocytes, suggesting that other SH3 domain-containing proteins than Hck and Lyn may also have functionally important interactions with Nef.

3.1.3. Lck

The role of Lck in mediating T cell activation as well as its binding to the intracellular tail of the CD4 have prompted studies on possible interactions between Lck and Nef even before the potential of Nef to bind to Src family SH3 domains was noted (72,81,82,121-123). Using various experimental strategies different groups have been able to detect Lck from T cell lysates, insect cell or bacterial extracts to associate with Nef. However, the mode by which HIV-1 Nef and Lck molecules interact remains controversial, and differing conclusions have been presented regarding whether the PxxP motif is dispensable for this interaction and if additional bridging molecules are required.

In any case, it is clear this interaction differs from that of the Hck/Nef complex in a number of ways. First,

while the binding of the Lck-SH3 domain to Nef may be involved, its affinity is modest compared to that of Hck-SH3 (57,109; M. Hiipakka and K.S., unpublished observations), and therefore the Nef/Lck complex must depend on other stabilizing contacts. These have been suggested to involve the SH2 domain of Lck (81,123) and/or additional bridging proteins (121). Second, while binding of Nef to the SH3 domain of Hck results in efficient activation of its catalytic function (92,114), as predicted based on the structural design of the Src kinases (115,116), it has been reported that Nef inhibits the catalytic activity of Lck (81,82). Third, Nef was reported to become tyrosine phosphorylated by Lck (81,121), whereas Nef was not found to be a substrate for tyrosine phosphorylation by Hck even though its kinase activity is strongly activated upon binding to Nef (92,114). Thus, if Hck and Lck both turn out to be important mediators of Nef function, despite their extensive structural and functional homology they appear to do so by very different molecular mechanisms,

3.1.4. More potential Nef-binding SH3 proteins

As discussed for Lck, it is conceivable that also low-affinity SH3 binding could be critical in coordinating and stabilizing interactions of Nef with host cell proteins if additional binding strength is be provided by other means. In this scenario, a large number of SH3 proteins serving in a variety of functions potentially involved in HIV cell biology can be added to the list of possible partners of Nef, because at least some affinity for Nef can be demonstrated for most of these proteins. For instance, Fyn, another member of the Src kinase family involved in T cell signaling has been found to bind Nef with a modest affinity (109,111,124,125).

Recently the guanidine nucleotide exchange factor (GEF) Vav was reported to interact with Nef (86). Vav is a multidomain 95 kDa protein consisting of Dbl homology (DH) domain, a pleckstrin homology (PH) domain, two SH3 domains and a SH2 domain, and functions as a GEF for the Rho family of small GTPases (126). The interaction between Nef and Vav (and also Vav2) was found to be mediated by the second SH3 domain of Vav, and dependent on an intact PxxP motif of Nef (86). Based on our semi-quantitative measurements binding of Nef to an isolated SH3-II of Vav also appears to be of low affinity and even weaker than binding to Fyn-SH3 (M. Hiipakka and K.S., unpublished observations). Nevertheless, the significance of the Nef/Vav interaction is suggested by biological consequences such as triggering of a JNK/SAPK signaling cascade and induction of cytoskeletal rearrangements in NIH3T3 cells upon co-transfection of Vav and Nef (86).

3.2. Nef associated serine kinases

It has been known for some time that Nef can be phosphorylated on serine residues, and associates with a number of cellular proteins that become trans- or autophosphorylated on serine or threonine residues in anti-Nef immunocomplexes *in vitro* (10,127-130). Serine phosphorylation of Nef has also been suggested to be involved in regulation of its activity (131,132). The degree of serine phosphorylation of HIV matrix protein in viral particles was also found to be increased in virions derived from Nef expressing cells (55). Such findings have prompted significant interest in identifying cellular serine kinases that might interact with Nef.

3.2.1. p21-activated kinase 2

Sawai *et al* first described in 1994 a phosphoprotein with molecular weight of 62 kDa observed in anti-Nef immunocomplexes after *in vitro* kinase reaction, and referred to this as Nef associated kinase activity (NAK) (133). Subsequently, several laboratories accumulated evidence suggesting that NAK may belong to the family of p21-activated kinases (PAKs) (73,134-136), but the identity of this kinase remained elusive until very recently, when it was conclusively identified as PAK2 (137).

Several regions in Nef have been found to be important for its interaction with NAK/PAK2. Within the core domain of Nef, the second residue (Arg107 in SIVmac239 Nef) of the conserved di-arginine (RR) motif has been found critical for PAK2 association (59,138). In the Nef/SH3 crystal structure this arginine residue is located on the edge of an exposed hydrophobic pocket of Nef in a prime position for being involved in coordinating an interaction with a Nef-binding protein (110). In cell culture studies the disruption of the Nef RR motif has been reported to lead to the loss of the ability of Nef to downregulate CD4, to increase HIV particle infectivity, and to block TCR-initiated signal transduction (30,59). It is not clear, however, if loss PAK2 interaction accounts for all these functional defects, or whether disruption of the RR motif affects functionality of Nef function in a more general manner.

Interestingly, also an intact PxxP motif has been found to be required for coprecipitation of PAK2 activity with Nef (59,139). The reason for the requirement of the PxxP motif which is located on the opposite side of the Nef molecule than the RR motif is not clear since PAK2 does not contain an SH3 domain. Another protein involved in the PAK2/Nef complex might contain an SH3 domain, which could stabilize this complex in a PxxP-dependent manner. The dependence of the interaction between Nef and PAK2 on other factors is also strongly suggested by the finding that NAK signal coprecipitating with Nef can not be increased by overexpression of PAK2 (137). Moreover, in addition to the RR and PxxP motifs, we have identified a third hydrophobic region on the surface of Nef, involving the residues Leu112 and F121 of the NL4-3 allele, which is required for its association with PAK2, and may represent the point of direct contact between these proteins (139).

Although the ability to bind to PAK2 is one of the most conserved functional features among the known HIV and SIV Nef alleles, the biological significance of this interaction is not yet clear. However, binding to PAK2 has been correlated with the ability of HIV-1 Nef to enhance viral infectivity as well as replication kinetics in primary cell culture (59,135), although this has been questioned in

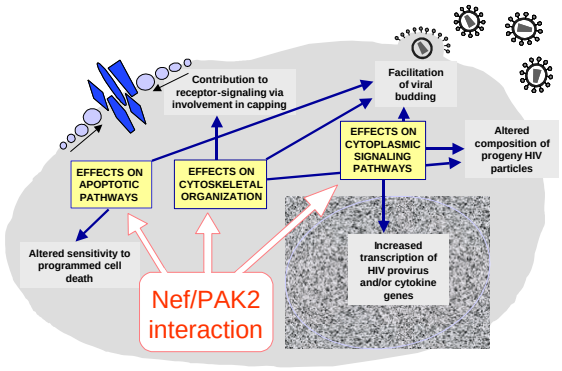

Figure 2. Possible roles for the Nef/PAK2 interaction in HIV life cycle.

the case of some Nef alleles (140). However, because Nef preferentially binds to an active form of PAK2 (G.H.R. and K.S., unpublished), a failure to detect Nef-associated kinase activity in some studies may have been caused by low levels of PAK2 activity in the cells examined, rather than a failure of the Nef allele used to associate with PAK2. Although Nef has been reported to activate the PAK family kinases via an effect on their upstream regulators (73), we have seen little evidence for this using native HIV-1 SF2 or NL4-3 alleles, and observe only low or undetectable levels of Nef-associated NAK/PAK2 activity unless cellular PAK-activity is induced by other means, such as cotransfection of an active form of a Rho-family GTPase (139).

Members of the PAK family serve important roles in mediating signals from plasma membrane to the nucleus, resulting in activation of transcription factors, such as the serum response factor, via a cascade of mitogen activated protein kinases (reviewed in refs 141-144). Furthermore, PAKs have direct effects on the cytoskeletal morphology, and PAK2 has been implicated in apoptotic signaling. It is possible that during HIV infection Nef could modulate the effects of PAK2 in mediating signaling from the cytoplasm into the nucleus and/or in inducing reorganization of the actin cytoskeleton, both of which could facilitate some step(s) in HIV life cycle, such as transcription, reverse transcription, or budding. The fact that PAK2, which Nef specifically selects as a partner, also is the only PAK family member that is cleaved and activated by pro-apoptotic caspases, suggests additional interesting possibilities to this list. For example, Nef/PAK2 interaction might modulate the sensitivity of HIV-infected cells to apoptosis, or alternatively, induce some apoptosis-like changes in the cellular physiology, which could favor HIV replication without causing an immediate destruction of the infected cells. These possible functions for the Nef/PAK2 interaction are summarized in Figure 2.

3.2.2. Other Nef-associated serine kinases

Nef has also been reported to associate with serine kinases that do not belong to the PAK family. Some of these have been suggested to represent members of the protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) families (see below). In most cases a direct physical interaction with Nef has not been shown, suggesting that their association with Nef might be mediated by one or several other factors involved in a common multiprotein complex. However, since most reports on these putative Nef-associated kinases discussed below represent only a single study that has not been followed up, very far-reaching conclusions regarding their role and significance as mediators of Nef functions are not yet possible.

Smith *et al*. have reported the identification of the 80 kD theta isoform of protein kinase C (θPKC) as a protein co-precipitating from Jurkat cell cytosolic lysates with GST-Nef fusion protein (145). Previous studies have shown that Nef can serve as a PKC substrate (129,130,132). However, by using a PKC pseudosubstrate peptide Smith *et al.* concluded that Nef/θPKC interaction was not mediated via the PKC substrate binding site. This interaction was reported to modulate the cellular activity of θPKC, since its normal relocation (unlike that of a panel of other PKC isoforms) to the particulate cellular fraction upon PMA/PHA stimulation was inhibited in Jurkat cells expressing Nef.

Baur *et al*. (121) described a cellular serine kinase in a multiprotein complex involving Nef and the tyrosine kinase Lck, that could serine phosphorylate these two proteins. Unlike NAK, this serine kinase activity was associated with the aminoterminus of Nef, and became evident in *in vitro* kinase assays only when Mn2+ was used as a divalent cation instead of Mg2+. The interaction is mediated by an amphipathic alpha-helix formed by Nef amino acids 16-22 whose predicted secondary structure is conserved among different Nef alleles. Deletion of the amino acids 16-22 resulted in loss of most of this Nef-associated serine kinase activity, and also greatly reduced the amount of co-precipitating Lck, suggesting that this serine kinase stabilizes the binding of Nef to Lck. In functional assays this N-terminal in-frame deletion caused an intermediate phenotype (as compared to Nef(+) and Nef (-) HIV in the PBMC replications assay, whereas the ability of the aa 16-22 deleted Nef to downregulate CD4 was not significantly affected. Preliminary data indicate that this serine kinase also is a PKC, but suggest it to be the delta rather than the theta isoform (A. Baur, personal communication).

Using a panel of antibodies to examine proteins that could be precipitated from cellular extracts with recombinant GST-Nef fusion protein, Greenway *et al.* reported one of these proteins to be reactive against an antibody for the mitogen-activated protein kinase $p44^{mapk/erk1}$ (72). Although Erk proteins do not have a SH3 domain, this interaction was found to depend on the Nef PxxP motif, (82). Thus, if binding to Erk1 proves to be a genuine function of Nef, it may be mediated or stabilized by another protein that contains an SH3 domain.

Hodge *et al*. (146) have reported that the c-Raf1 kinase, which plays an important role in coordinating the Ras-Raf-MAPK pathway, can be co-precipitated from CEM T-cells together with Nef. This complex may involve

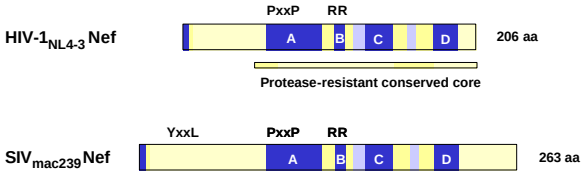

Figure 3. Comparison of the primary structures of Nef proteins encoded by HIV-1 NL4-3 and SIVmac239. The degree of amino acid conservation is indicated by the coloring, the pale yellow areas thus representing regions with little or no sequence homology between HIV-1 and SIV Nef. The dark blue "Nef boxes" A, B, C, and D, as defined by Shugars *et al.* (164), represent blocks of residues which are particularly well-conserved among Nef proteins from different primate lentiviruses. Residues outside these boxes may, however, be highly conserved within one of these virus families. The locations of the YxxL, PxxP, and RR motif discussed in the text are shown. The protease-resistant core fragment used for X-ray crystallography, and corresponding to the HIV protease cleavage product observed in virions (48,50) is indicated by the small bar.

a direct interaction between these proteins, as these authors also demonstrated that recombinant Nef and Raf produced in *E. coli* could interact. This binding was dependent on a conserved di-aspartate motif in Nef, which was proposed to resemble an acidic consensus Raf-binding motif previously characterized in Ras. In previous studies the same conserved acidic motif has been shown to be required for CD4 downregulation by Nef (30,32), and suggested to mediate this function by binding to the catalytic subunit of vacuolar ATPase (25)

3.3. T cell receptor zeta chain

Using yeast two-hybrid screening two laboratories have independently reported a direct association of SIV Nef with the zeta chain of the T cell receptor (TCRζ) (147,148). In these studies mutations of the PxxP and RR motifs had little effect on the association with TCRζ (148), and binding to SIV and HIV-2 but not HIV-1 Nef proteins was detected (147). More recently, Xu *et al.* reported that also a membrane targeted form of HIV-1 Nef could interact with TCRζ, and that this interaction was dependent on a functional PxxP motif of Nef (95). Thus, it is possible that both SIV/HIV-2 and HIV-1 Nef associate with TCRζ, but that the latter accomplish this in a more complex manner, perhaps assisted by cellular SH3-containing proteins.

Because TCRζ serves as a critical intracellular effector of TCR signaling, it is easy to envision how binding of TCRζ could serve as a physical link in Nef-mediated modulation of the TCR-response (whether positive or negative), or induce TCR-regulated events in the absence of TCR activation. Indeed, the study by Xu *et al.* indicated a critical role for the Nef / TCRζ interaction in Nef induced FasL expression (95).

3.4. How similar are HIV and SIV Nef proteins?

SIV infection of rhesus macaques has been regarded as the ultimate test for the functionality of different Nef alleles, and is the model in which the requirement for Nef in the pathogenesis of AIDS was first demonstrated (1). In this model, unlike in cell culture systems or in SCID-hu mice, the interplay between the virus and the immune system can also be assessed. While important in any infectious disease, this might be particularly relevant to appropriately reveal the *in vivo* function of Nef. The possibility to examine the reversion of mutations in Nef that inhibit the replicative potential of the virus is another valuable feature of this model.

Unfortunately, a number of recent publications have suggested that the value of the SIV/ macaque model in testing predictions derived from *in vitro* work on HIV-1 Nef is limited due to differences in the molecular mechanisms of action of HIV-1 and SIV Nef proteins. Such differences were unexpected, because although regions of substantial amino acid dissimilarity exist in these proteins (see Figure 3), the Nef regions that are highly conserved among different isolates of HIV-1 are also similar in SIV. More important, the panel of cellular effects of Nef expression are very similar regardless of whether HIV-1 or SIV Nef proteins are used in these experiments (149,150, and studies discussed in Chapter 2). Nevertheless, it has become clear that these homologous proteins may employ divergent molecular strategies to carry out their similar functions, even when these functions involve the same target molecules of the host cell.

The differential binding of SIV and HIV-1 Nef to TCRζ discussed above provides one example of this. Other examples of such functional differences also point out to a lesser reliance of SIV Nef on interactions mediated by the SH3-ligand (PxxP) motif. Whereas the effects of HIV-1 Nef on cellular signal transduction, and the binding to the Src-family kinases strictly depend on an intact PxxP motif, disruption of this motif in SIVmac239 Nef had only a minor effect on its ability to block TCR-initiated signal transduction (30), and this mutant retained the capacity to still bind and activate Src kinases via an SH3 independent mechanism (151,152). It therefore appears that SIV Nef can utilize alternative or redundant strategies for modulating cellular signaling pathways. A similar situation is seen also in the case of CD4 downregulation (24, 30,153-155). SIV Nef contains two tyrosine-based sorting signals that are important for its ability to down-regulate CD4, and to associate with the μ chains of clathrin adaptors. These tyrosine motifs are not present in HIV-1 Nef, which instead depends on a leucine-based motif in targeting CD4 for accelerated endocytosis. However, a similar leucine-based motif is also present in SIV Nef, which thus differs from HIV-1 Nef by utilizing two parallel pathways of the protein-sorting machinery for CD4 downregulation.

A series of recent publications have shown that it is possible to substitute the disease-promoting function of SIV Nef with that of HIV-1 Nef, and observe high viral loads and development of simian AIDS in macaques infected with such chimeric SHIV viruses (156-158). These promising results suggest that the above-discussed problems

could be circumvented in using the macaque model for testing the *in vivo* relevance of different functional properties of HIV-1 Nef, and for guiding the development of novel antiviral therapies directed against HIV-1 Nef.

It remains to be seen, however, to what extent the functional properties of the HIV-1 Nef alleles of such pathogenic SHIV strains have changed by the mutations that have accumulated during their adaptation for optimal growth in macaques. Of note, a mutation that was consistently noted to occur in the HIV-1 SF33 Nef allele used by Luciw and colleagues (157), provided it with a novel YxxL sequence resembling the tyrosine-based motif, which is employed by SIV Nef for CD4 downregulation but is not normally present in HIV-1 Nef. Thus, the differences in the molecular mechanisms of action of SIV and HIV-1 Nef proteins might not just represent alternative strategies adopted by these viruses since their evolutionary divergence, but could indicate some fundamental differences in the virus-host relationship in human AIDS versus SIV infection of macaques.

4. PERSPECTIVE

Published evidence of modulation of host cell signaling cascades by the lentiviral Nef proteins continues to accumulate. In agreement with the idea that during the early part of the intracellular phase of HIV/SIV life-cycle Nef expression would contribute to host cell activation, salient examples of positive effects by Nef on cellular signal transduction have also been documented. Interestingly, in some cases such stimulatory effects have been shown to involve auto- or paracrine mechanisms (90,91). However, understanding how the physical and functional interactions of Nef with different host cell signaling molecules contribute to its multiple cellular effects, and ultimately to Nef-dependent disease progression *in vivo* still pose major challenges for future research.

The number of Nef-associated host cell proteins is large and growing (Table 2). Although Nef appears to function as an adapter molecule, the list of its potential cellular partners seems excessive. In order to clarify this situation, it will be important to determine which of these proteins bind to Nef directly, and subsequently carefully characterize these interactions using quantitative biochemical methods and approaches of structural biology.

In addition to contributing to development of high viral load and associated immunodeficiency in infected individuals, Nef has been shown to be independently able to induce AIDS-like pathology in transgenic mice (2). This two-fold role of Nef in progression of AIDS suggests that therapeutic approaches targeting Nef could be an efficient addition to the current antiretroviral therapy for HIV infection. The increasing understanding of the molecular mechanisms involved in Nef function, and the recent developments in animal models for HIV-1 Nef-induced disease, such as pathogenic $SHIV^{Nef}$ viruses and Nef-transgenic mice, provide hope that development of anti-Nef drugs may be feasible.

5. ACKNOWLEDGEMENTS

This work was supported by grants to K.S. from the Academy of Finland (SA152304) and the Medical Research Fund of Tampere University Hospital. G.H.R. is an EU Biomed Marie Curie program Fellow.

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Key words: Nef, HIV, SIV, AIDS, protein kinase, SH3 domain, Review

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