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HIV-1 Nef AND HOST CELL PROTEIN KINASES

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TABLE OF CONTENTS

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). Notably, animals infected with Nef-deleted attenuated viruses are resistant to subsequent challenge with pathogenic wild-type viruses (2) A critical role for Nef in development of AIDS in humans has been suggested by the observation that some individuals with a long-term nonprogressive HIV-1 infection (persons who show no clinical or immunological signs of immunodeficiency despite being HIV seropositive for over a decade) are infected with viruses carrying naturally occurring Nef deletions (3, 4). 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 protein kinases. These findings will be discussed in the following, preceded by a short introduction into the role of Nef in cell biology of HIV infection, which is intended to serve as a critical review of our current understanding on this enigmatic issue rather than a comprehensive review of the literature.

2. PHENOTYPIC EFFECTS OF Nef EXPRESSION

The growth 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 (5, 6). Another seemingly related phenotype reported for Nef in cell culture is its ability to increase the intrinsic infectious potential of HIV particles in single-round infection or end-point dilution assays (5). Interestingly, the lower infectivity of HIV particles which 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 (7-11). This increased infectivity is not related to the success in entering into the target cells, but becomes apparent upon examination of the efficiency in completion of the reverse transcription of the viral genome.

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It has been suggested that the mechanism by which Nef increases HIV infectivity involves packaging of Nef protein into the progeny virus particles. Indeed, an average of 10-100 Nef molecules per virion has been shown to be present in HIV particles, where most of it is cleaved by the HIV protease between the membrane-anchoring aminoterminus and the conserved Nef core domain (12, 13). However, the significance of the presence or cleavage of Nef in the virions remains to be demonstrated, and has been called into question by findings on behavior of certain mutant forms of Nef (14; H-G. Kräusslich, personal communication). Alternatively, if Nef protein incorporated into the virions is not important, one would have to postulate that in order to enhance particle infectivity Nef must somehow modify other viral proteins or cell-derived components of the virions. To this end, it has been suggested that expression of Nef increases the incorporation of a cellular serine kinase into the virions (15). On the other hand, decreasing the incorporation of certain host cell surface proteins into the envelopes of budding viruses could conceivable also be involved in increasing HIV particle infectivity.

Downregulation by a posttranslational mechanisms of the cell surface expression of the CD4 receptor for the virus (16-19) as well as the major histocompatibility complex class I (MHC I) molecules (20, 21) are two other known phenotypic effects attributed to Nef *in vitro*. Downregulation of CD4 requires a dileucine motif in its intracellular tail, and results in increased endocytosis and transport of CD4 into the lysosomal compartment in a manner that is independent of CD4 serine phosphorylation which normally triggers this event (20, 21). Downregulation of MHC I molecules, which do not contain a dileucine motif, also results in their accumulation in the lysosomes (21), but it is unclear how similar these two Nef-induced events are mechanistically. Unlike CD4 and MHC I, a number of other proteins whose cell surface expression is also regulated by endocytosis are not affected by Nef, indicating that this effect is relatively specific (21, 22). It has been speculated that downregulation of CD4 might enhance HIV replication by facilitating the release of virus progeny or by preventing a potentially hazardous superinfection of cells. On the other hand, this could be

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

- Increased infectivity of viral particles.
- Enhanced viral gene expression.
- Modulation of apoptosis.
- Evasion from CTL response.
- Altered Th1/Th2 balance.
- Decreased expression of inhibitory cytokines or chemokines.
- Increased number of new target cells by activation of bystander cells.
- Facilitation of the budding of viral progeny.
- Prevention of cells from superinfection.

an unimportant by-product of some other more consequential event, for example liberation into the cytoplasm of Lck protein tyrosine kinase that is normally bound to the intracellular tail of CD4 (22-24). In the case of MHC I, suggested potential pathophysiological roles for its downmodulation by Nef involve decreasing the amount of MHC I molecules that get incorporated to the envelopes of virus progeny and perhaps more intriguingly, escape of HIV-infected cells from recognition and elimination by cytotoxic T cells.

It has been shown that downregulation of both CD4 and MHC I is unrelated to the Nef-induced increase in HIV particle infectivity (5, 20) as particles produced in cells lacking CD4 or MHC I also display this Nef-dependent phenotype. Moreover, we have demonstrated (25), and a number of studies have subsequently confirmed (26-29), 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 later).

The failure to complement the lower infectious potential of Nef-deleted viruses by expression of Nef in the target cell of infection, the demonstrated facilitating effect of Nef on a step in retroviral life-cycle that occurs before de novo synthesis of viral proteins (including Nef), plus similar yields of virus obtained from cells transfected with HIV proviral plasmids regardless of whether they carry a functional Nef gene, have collectively led to an idea that Nef is a protein which is required during the very last steps of HIV life-cycle (virus particle formation) but manifests its function in the target cell during an early post-entry step before proviral integration. However, it would probably be unwise to ignore the fact that the elaborate sequential pattern of gene expression which primate lentiviruses have evolved to utilize (reviewed in ref. 30), appears to point to the opposite, suggesting a principal role for Nef during the early post-integration stage of HIV infection when it constitutes the majority of all viral proteins synthesized.

It is likely, however, that Nef has multiple functions which are important in distinct steps of viral life-cycle and in different aspects of HIV pathophysiology. A list of possible and partially overlapping pathogenic mechanisms, mainly based on extrapolation from the *in vitro* findings discussed elsewhere in this review, which might be involved in the Nef-induced HIV/SIV disease progression *in vivo* is presented in table 1.

Although it is probable that the critical *in vivo* function(s) of Nef is among or related to the listed mechanism, it is at the present difficult to speculate on the relative pathogenic importance of these scenarios. While the effect on HIV particle infectivity - perhaps the currently favored model of Nef action - may indeed be a significant function of Nef, there is no data to indicate that this would be crucial for its role in promoting disease progression *in vivo*. In fact, some observations hint to the possibility that such Nef-induced increase in HIV particle infectivity may not even account for the enhanced growth kinetics of Nef+ viruses compared to Nef- viruses in culture. For example, while the enhanced replicative kinetics provided by Nef can typically only be observed using the primary cell system described above, the increased HIV infectivity by Nef can be readily demonstrated with a number or transformed producer/target cell combinations. In this regard it is curious why successive rounds of production of apparently 5-to-50-fold more infectious virus do not amount to enhanced replication kinetics of Nef+ viruses in most cells. Also, although available parallel comparison data is scanty, the positive effect of Nef on single-round HIV infectivity appears to be much more dependent on the HIV strain used than the enhancing effect on viral growth kinetics in PBMCs. Furthermore, certain mutations made to Nef seem to affect these two properties in an uneven manner (H.-G. Kräusshlich, personal communication). One interesting possibility is that the positive effect of Nef on viral growth kinetics in PBMCs is relatively indirect, and perhaps related to the recent observation by Desrosiers and colleagues who reported that the substantial growth advantage of Nef+ SIV in H. saimiri-immortalized IL-2-dependent T cell clones correlates with endogenous IL-2 production induced by Nef in these cultures (31).

Such an indirect mechanism for Nef action on viral replication through altered host cell physiology is supported by the demonstrated potential of Nef to regulate cellular signal transduction pathways. 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 (32). More recently, HIV Nef was shown to cooperate with a cellular proto-oncogene product, protein tyrosine kinase Hck, in transforming another immortalized fibroblast cell line, Rat2 (33). Interestingly, this appears to involve deregulation by Nef of the normally tightly controlled kinase activity of Hck (see later). 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 been reported 28, 32, 34-47; A. Manninen and K.S., unpublished results). It is, however, not easy to build a coherent picture from these observations. One major problem is that often positive, negative, or lack of any effects have been reported regarding the same signaling step. In addition to the molecular clone of Nef used in these studies, such differences might be attributed to a number of variables, including the selection of the cell line and culture conditions, or the means, level and duration of Nef expression, all of which could profoundly modify the cellular responses to Nef due to their general effects on cellular physiology or specific effects on the activity or relative abundance of cellular partners of Nef. In line with such reasoning Baur and colleagues have reported that depending on experimental conditions the subcellular distribution of Nef may be different (plasma membrane vs. cytoplasm), and this can result in contrasting effects on cellular activation markers such as protein tyrosine phosphorylation and the activity of the transcription factors NF-kappaB and AP-1 (36).

Despite these unresolved issues, alteration of cellular signal transduction is a well-documented function of Nef, and an attractive candidate to explain its pathogenic effects *in vivo*. Modulating cellular activation could not only lead to increased virus replication and infectivity of viral particles produced in the infected cell, but could also promote spreading of

Table 2. Putative cellular Nef-binding proteins

Protein kinases
- Src family tyrosine kinases
  - Hck (25, 33, 48-50)
  - Lyn (25)
  - Lck (42, 51-53)
  - Src (31, 32)
- PAK family serine kinase (29, 43, 54-57)
- Protein kinase C theta (58)
- Protein kinase C delta (A. Baur, personal communication)
- Mitogen-activated protein kinase Erk-1 (42, 51)
- Unspecified serine kinase(s) (59)

Other proteins
- CD4 (42, 60-62)
- p35 thioesterase (63; G. Cohen, personal communication)
- beta-COP (64)
- Actin (65)
- Adaptins (66; D. Trono, personal communication)

HIV to neighboring cells through their paracrine stimulation. By altering the physiology and antiviral responses in the infected cells Nef could conceivably also modulate apoptosis in these as well as in cells involved in antiviral defense, or affect the balance of cytokine networks of the immune system in ways that may promote progression of the infection (table 1).

3. CELLULAR PARTNERS AND TARGETS OF Nef

A key to understanding how Nef mediates its intracellular functions is to identify the immediate cellular partners of Nef which it physically interacts with. Indeed, a large number of cellular proteins have been suggested to bind to Nef (table 2). None of these, however, have so far been conclusively demonstrated to play a role in the pathogenesis of AIDS in humans or other primates, and we are only beginning to understand how some of them might be involved in the known phenotypic effects of Nef in cell culture.

The candidate Nef-binding cellular proteins listed in table 2 have been identified by diverse experimental strategies, and are classified under two broad categories, namely “protein kinases” and “other proteins”. It is of interest to note that most of the proteins in the latter category, i.e. proteins that do not possess kinase activity, are in one way or another implicated in downregulation of CD4 cell surface expression. In addition to CD4 itself, whose apparently low-affinity direct interaction with Nef (42, 60-62) has been a controversial issue, these include beta-COP (64), a component of the cellular endocytic apparatus, as well as a novel type of mammalian thioesterase which was identified independently by yeast-two hybrid screening and Nef-affinity purification, and shown to be involved in CD4 downregulation through the use of Nef mutants that fail to bind to it (63; G. Cohen, personal communication). Recently, adaptins, proteins involved endocytosis by connecting receptors with clathrin-coated pits, have also been found to be capable of physically interacting with Nef (D. Trono, personal communication).

Conversely, all the other putative Nef-binding proteins in table 2 are protein kinases involved in cellular signal transduction. Of these, the Src family of tyrosine kinases and a 62/72 kD Nef-associated serine kinase (NAK) which is believed to belong to the p21-activated kinase (PAK) family, have been studied most intensively (see references in table 2). Interestingly, the binding of both classes of kinases to Nef is dependent on an intact proline-repeat (PxxP) motif in Nef (25, 29) (see discussion later). Similarly, many effects of HIV Nef on cellular signal transduction, such as inhibition of T cell receptor (TCR) initiated CD69 induction (28), have also been found to depend on the same conserved proline motif of Nef. On the other hand, although the PxxP motif is critical for the enhancing effect of Nef on HIV replication kinetics and particle infectivity, it is dispensable for CD4 downregulation (25). Based on these observations it is therefore tempting to generalize that the effects on cellular signaling and on HIV replication may belong to the same and mechanistically related category of Nef functions, while CD4 downregulation (and perhaps MHC I downregulation) may belong to another, which does not involve components of cellular signaling cascades.

3.1. Nef associated tyrosine kinases

3.1.1. Introduction into the Src family of tyrosine kinases

The possibility that Nef might interact with Src family tyrosine kinases was suggested by the identification of a proline repeat motif P-x-x-P (where x is any amino acid) as the minimal consensus sequence defining the ligands of SH3 (Src homology 3) domains (67, 68). 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 (69). In addition to Src itself this kinase family consists of eight known members, Blk, Fyn, Fgr, Hck, Lck, Lyn, Yes, and Yrk (for reviews, see refs. 70 and 71). Src was originally discovered as the cellular counterpart (proto-oncogene) of the transforming gene of Rous sarcoma virus (v-Src oncogene). An important function of the Src kinases is to relay signals from outside of the cell which are mediated by transmembrane proteins which lack independent catalytic activity. The PxxP-binding SH3 domain together with the SH2 domain, a modular protein unit which binds to tyrosine phosphorylated target peptides, not only mediate the interactions with the cellular partners and ligands of the Src proteins but also serve to negatively regulate their catalytic activity by engaging in similar intramolecular interactions (69, 72, 73).

3.1.2. SH3 / Nef interaction

The structural basis of the SH3/PxxP interaction is now well-understood (69, 74, 75). The PxxP containing peptides adopt a secondary structure known as polyproline type II (PPII) helix, in which exactly three residues constitute one left-handed helical turn, thus placing the two PxxP-defining proline residues adjacent to each other one side of the helix. 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, in particular with a set of aromatic residues which are highly conserved among different SH3 domains.

Interestingly, the pseudosymmetric nature of the PxxP helix enables it to dock on the SH3 domain in two opposite orientations (figure 1), defined by the pattern of other strictly conserved hydrophobic and positively charged residues in the PxxP-peptide. In most SH3 ligands the presence of an arginine or lysine (R in figure 1) before or after the two prolines marks the orientation of the binding (+ or -) by forming a salt bridge with a negatively charged residue which is present in the corresponding position in all Src family members as well as in most other SH3 domains. Although these molecular details were not yet elucidated at the time when HIV-1 Nef was first shown

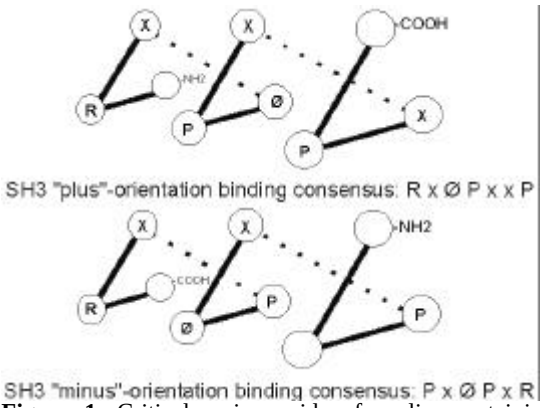

Figure 1. Critical amino acids of proline-containing SH3 ligands mediating their binding to SH3 domains in two opposite orientations ("plus" and "minus"). The figure is drawn based on Lim *et al*. (74) and Feng *et al*. (75), as well as a number of studies defining optimal SH3-binding peptides in naturally occurring ligands or peptide libraries (reviewed in ref. 69). R = arginine, P = proline, Ø = hydrophobic aliphatic amino acid.

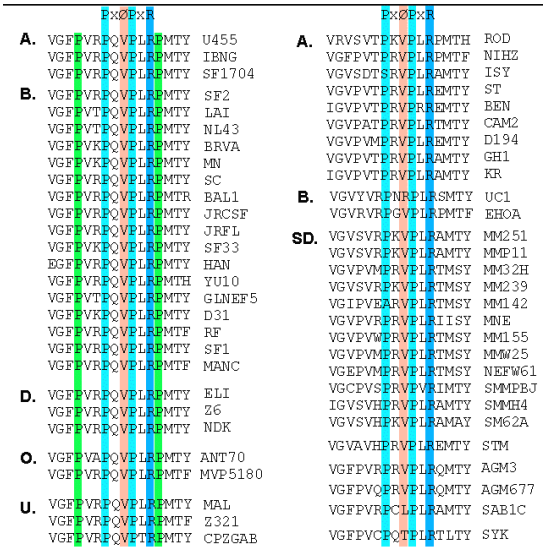

Figure 2. Evolutionary conservation of a minus-orientation SH3 binding site in Nef sequences from different primate lentiviruses. The sequence PxØPxR (where x is any amino acid and Ø is a hydrophobic aliphatic residue) is, besides the HIV-1 Nef sequences shown (left panel), conserved in 70/70 HIV-1-like 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.

to bind to the SH3 domains of a subset of Src kinases, it is noteworthy to point out in retrospect 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. All 70 HIV-1 Nef amino acid sequences and a great majority of those of HIV-2 and SIV available in the Los Alamos database contains this motif (figure 2).

Mutagenesis 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 (48). 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. The affinity of Nef to Fyn is less than 1% of that of the Hck/Nef interaction, despite the conservation between Hck and Fyn of virtually all of the residues that accommodate and coordinate the binding to the PxxP peptide (48). Recent X-ray crystallographic determination of the structure of the core region of Nef complexed with an SH3 domain has provided a detailed look into the such tertiary interactions, and again revealed the involvement of highly conserved residues in Nef, in particular two 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 (figure 3; 49).

3.1.3. Role of the PxxP motif *in vivo*

The strong sequence conservation of the Nef PxxP motif among different primate lentiviruses together with the functional data on mutations that disrupt it, suggest an important role for this motif in mediating the pathogenic functions of Nef. In addition to its enhancing effect on HIV-1 replication kinetics and particle infectivity in cell culture, the PxxP motif has also been found to be important for higher pathogenic potential of Nef-carrying viruses in the SCID-hu mouse model for HIV-1 infection (Aldrovandi and Zack, submitted). In the light of these findings, it is surprising that conflicting results have been observed in two recent studies regarding the requirement for the Nef PxxP motif in development of simian AIDS in SIV-infected macaques (76; P. Luciw, personal communication).

Although both studies found evidence for a selective advantage for the PxxP-containing viruses, as indicated by the reversion of mutations made to change these residues into alanines, only Luciw and colleagues concluded that such reversions are absolutely necessary for development of simian AIDS. By contrast, Lang *et al*, (76) reported on rapid (9 and 16 weeks) progression to disease in both animals which they infected with a PxxP-to-AxxA mutated viruses before significant (>10%) proportion of sequenced proviruses showed reversions in these proline-codons. Although, given such rapid disease progression, a higher number of reversions would probably have been seen among the infectious plasma virus population than in the PBMC proviral DNA pool which represents more "archival" material, this study markedly contrasts to the results obtained by Luciw and co-workers who infected a larger number of macaques (n=6) with SIVmac239 viruses carrying a similar Nef PxxP-to-AxxA change. One monkey died rapidly (19 weeks) of a disease that did not resemble simian AIDS, and an abnormally high blood eosinophil count observed at the time of infection suggested involvement of an underlying predisposing condition in this animal. In all of the remaining five infected animals the virus load was initially as low as in animals infected with a Nef-deleted virus, and increasing viremia and subsequent disease progression (seen in 4 animals) was invariably associated with reversion of the PxxP-defining prolines. Of the two PxxP-defining prolines the second one appeared to show a stronger selective advantage, and reverted earlier and in a greater proportion or the total number of sequenced clones.

The reasons for the discrepancies between these two studies are not clear, but could be related to genetic or environmental factors which might inluence the relative necessity of a fully functional Nef gene for disease progression in the SIV-infected animals. Since it appears that the functionality of SIV Nef may be less severely compromised upon disruption of its PxxP motif than is HIV-1 Nef (28), such differences could well be

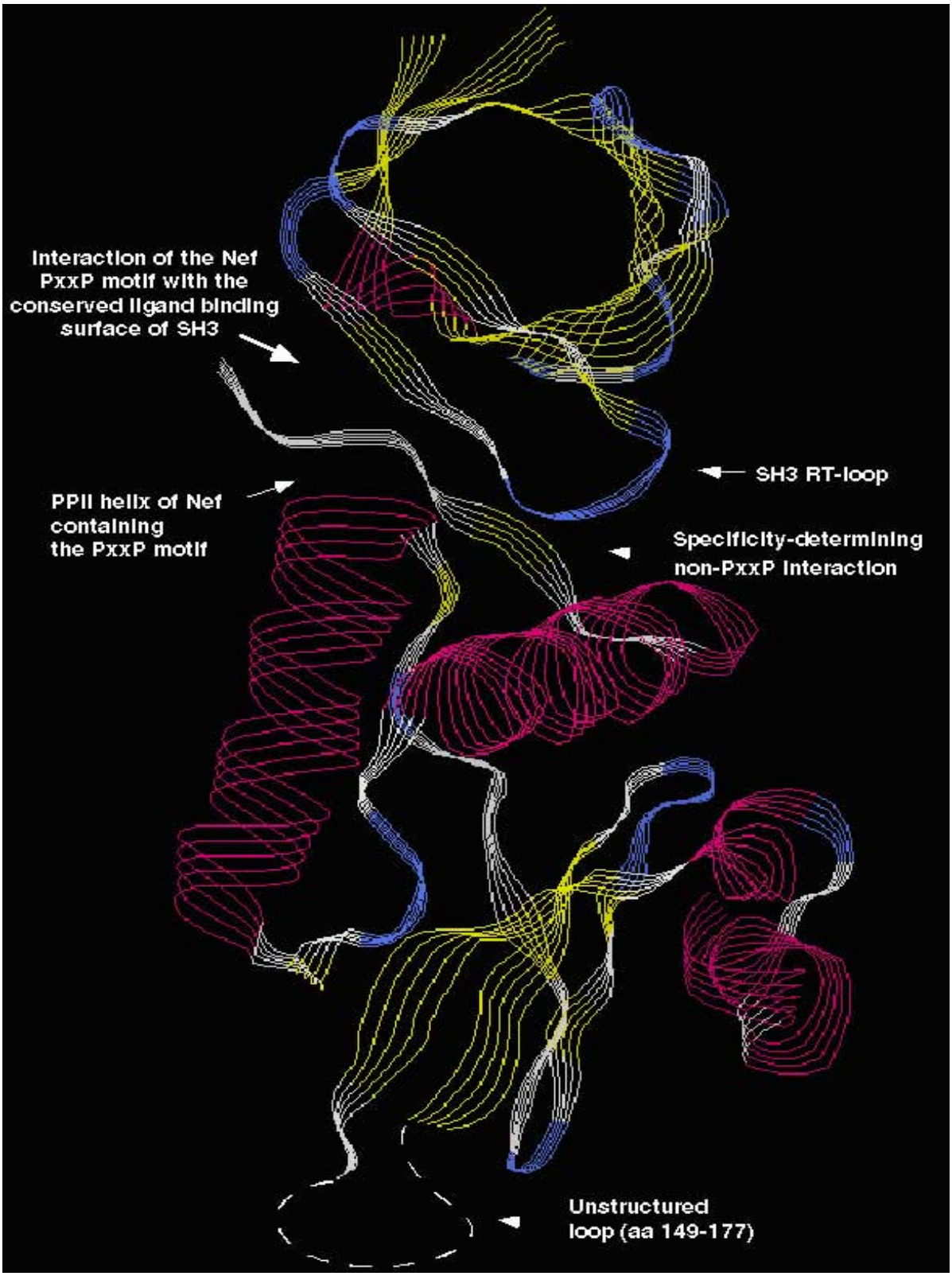

Figure 3. Structure of the conserved core domain of HIV-1 Nef (amino acids 54-205) complexed with an SH3 domain (49). The region preceding the PPII helix (amino acids 54-70) as well as a loop (amino acids 149-177) connecting the third and fourth beta sheets were disordered in the crystals, and are not included in the model. For structural determination a mutated Fyn-SH3 was used, in which an arginine residue (R96) normally present in the RT-loop of Fyn-SH3 was replaced with an isoleucine to provide it with Hck-like affinity for Nef. The figure was created based using the RasMac v2.5 molecular graphics program. Alpha helices are coloured magenta, beta sheets yellow, turns pale blue, and all other residues are coloured white. The corresponding coordinates have been deposited in the Brookhaven Protein Data Bank (ID: 1efn)

envisioned to have a significant impact on the outcome of the infection. It should be remembered, that even delta-Nef viruses are not completely non-pathogenic, and have been reported to cause disease upon infection of newborn macaques (77), and in some cases may do so in adult animals as well (78). It may therefore be worth noting that the study by Lang *et al*. (76) only involved two macaques, and that the rapid development of disease in these animals, particularly in the case of the monkey which had to be sacrificed as early as 9 weeks after the infection, is not typical for simian AIDS.

3.1.4. 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 the tightest SH3/ligand interaction so far reported (48). 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 (25; 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 (48). Therefore, although the following discussion focuses on the possible role of Hck in the pathogenesis of AIDS, many of the same arguments may 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.

The SH3-mediated interaction between Hck and Nef can be demonstrated by their co-immunoprecipitation from cells in which both proteins are expressed (33). Remarkably, this interaction has striking functional consequences in such cells, leading to their malignant transformation. The mechanism of co-transformation by Nef and Hck was shown to involve deregulated growth signaling caused by enzymatic activation of Hck. By contrast, no Nef/Hck complexes, activation of Hck, or malignant transformation was seen when a PxxP-defective mutant of Nef was used, indicating a role for SH3-binding in these phenomena (33). Although a remarkable result, the transforming potential of coexpressed Nef and Hck was not completely unexpected. It has been known that certain mutations in the SH3 and SH2 domains of Src kinases can result in their catalytic activation and render them transforming (79, 80). On the other hand, it was recently 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, capable of overriding other mechanisms of negative regulation (50). The recently published three-dimensional structure of an almost complete Hck protein (72), together with a similar structure of Src (73) and a structure of an active catalytic domain of Lck (81), provide a sound 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 above-discussed biochemical, cellular, and functional studies make Hck an attractive candidate for a cellular accomplice of Nef. However, the pattern of Hck expression suggests that its possible 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 (71). 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 probably have to somehow facilitate HIV infection in the T cell compartment. Because most T cells in the body are resting and therefore resistant to HIV infection (unlike monocyte/macrophages) production of T cell-activating cytokines (such as IL-1) or other stimulatory molecules (such as HIV Tat) might be involved. 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 T cells which they meet.

On the other hand, myeloid cells have also been directly implicated as important reservoirs for HIV replication in their own right 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 (82, 83). 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 (84).

Despite such considerations, however, 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 (or Lyn) may also have functionally important interactions with Nef. The unusual compatibility of Hck-SH3 in binding to Nef, and the striking functional consequences of this interaction suggest a role for a yet unidentified protein closely resembling Hck in these properties. However, such a protein remains to be identified, and other scenarios should also be considered.

3.1.5. Lck and other SH3 proteins

The role of Lck in mediating T cell activation as well as its physical association with the intracellular tail of the CD4 have prompted studies on possible interactions between Lck and Nef well before the potential of Nef to bind to certain Src family SH3 domains was noted. Despite the long-lasting experimental interest in this possibility, positive findings on an association between Nef and Lck have been reported only recently (51-53). Using different experimental strategies three different groups have reported small but detectable amounts of Lck from T cell lysates coprecipitating with Nef, or *vice versa*. Despite the seeming congruence of these studies, however, distinct differences in these reports are also apparent. Collette *et al*. (52) suggested that Lck can directly associate with Nef without the assistance of other factors via a PxxP/SH3-domain interaction that is strengthened by an additional contact involving the SH2 domain of Lck. The proposed role for the SH2 domain suggests that tyrosine phosphorylation of HIV Nef may also be involved. On the other hand, Baur *et al*. (53) reported that Nef binds to Lck via its aminoterminal

region, although they suggested that additional cooperating interactions might also be provided by the central PxxP-containing region of Nef. Furthermore, Baur and colleagues reported that the Nef/Lck interaction is critically dependent on the presence of additional proteins derived from Jurkat cell lysates, one of which appears to be a serine kinase that can phosphorylate both Nef and Lck in these complexes (see later).

Although it is probably still too early to make definitive conclusions from these studies, one puzzling observation seems to be relatively obvious. If Hck and Lck both turn out to be important mediators of Nef function, they appear to do so in very different molecular mechanisms, despite the extensive structural and functional homology shared by these two Src family kinases. First, while the binding of the Lck-SH3 domain to Nef may be important, its affinity is modest compared to that of Hck-SH3 (25, 48; M. Hiipakka and K.S., unpublished observations), and therefore probably must depend on other stabilizing interactions or a protein complex. Second, while binding of Nef to the SH3 domain of Hck results in efficient activation of its catalytic function (33, 50), as predicted based on the structural design of the Src kinases (72, 73), it has been reported that Nef would interfere with the catalytic activity of Lck (51, 52). Third, despite such inhibition, Nef was reported to become tyrosine phosphorylated by Lck (52, 53), 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 (33, 50).

Besides Hck, Lyn, and Lck, other Src family members or other SH3 domain-containing proteins have not been implicated as cellular partners of HIV-1 Nef. Src itself has been reported to bind to SIV Nef, but this interaction appears to depend on a region unique to SIV and missing from HIV-1 Nef (see later). However, as suggested for the Nef/Lck interaction (52), it is conceivable that also low-affinity PxxP-SH3 contacts could be critical in coordinating and stabilizing interactions of Nef with proteins or protein complexes, in which the principal binding affinity would be provided by other means. If such scenarios are also considered, 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, as a low affinity for Nef can be demonstrated for most of these proteins, such as Fyn (48).

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 residues in anti-Nef immunocomplexes *in vitro* (18, 85-88). Serine phosphorylation of Nef has also been suggested to be involved in regulation of its activity (89, 90). Recently, 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 (15). Such findings have prompted significant interest in identifying cellular serine kinases that might interact with Nef.

3.2.1. Nef-associated 62/72 kD kinase (NAK)

Sawai *et al* first described two phosphoproteins with molecular weights of 62 kD and 72 kD observed in anti-Nef immunocomplexes after *in vitro* kinase reaction, and referred to these as Nef associated kinase activity (NAK) (54). More recently, Nunn and Marsh found that the 62 kD form (described by them as p65) was able to bind to an ATP analog as well as phosphorylate myelin basic protein and histones *in vitro*, indicating that it is indeed a *bona fide* protein kinase (57). It is possible, that the 72 kD component of NAK which is only found from T cells, represents a substrate for serine phosphorylation rather than a kinase.

A clue to the identity of NAK was provided by the observation that the corresponding 62/72 kD polypeptides could also be immunoprecipitated from Nef-expressing Jurkat T cells using antibodies against the p21-activated kinase (PAK) family member PAK1 (56, 57). The PAK family of serine/threonine kinases is regulated by the Rho-family GTPases Cdc42 and Rac, and believed to serve an important role in mediating signals from plasma membrane to the nucleus, resulting to activation of transcription factors, such as the serum response factor, via a cascade of mitogen activated protein kinases (reviewed in ref. 91). Interestingly, such autophosphorylated 62/72 kD NAK polypeptides were not seen in anti-PAK1 precipitates from Jurkat cells which did not express Nef, suggesting that Nef is not only capable of associating with NAK, but also induces its catalytic activation (56). Whether this activation is a direct consequence of Nef binding to NAK, or if Nef causes it via a more indirect mechanisms is not clear. The appearance of activated (autophosphorylated) NAK in Nef-immunocomplexes has been shown to be increased by cotransfected dominantly active Cdc42 or Rac but decreased by dominant negatively acting forms of these GTPases or a dominant negative mutant of hPAK65 (43). The ability of NAK to phosphorylate histone H4 *in vitro* has also shown to be increased in the presence of Cdc42 (57). Together these observations provide evidence that NAK is not only serologically but also functionally related to PAKs. Although the *in vitro* phosphorylated p62 and p72 components of NAK in Nef-immunocomplexes were found to share similar partial proteolytic phosphopeptide maps with the corresponding phosphoproteins precipitated directly with the anti-PAK antiserum (56), they both differed in such an analysis from a constitutively active form of PAK1 expressed in the same cells (57). Also, NAK does not seem to be identical with either one of the two other known PAK kinases, PAK2 or PAK3 (57). Thus, detailed biochemical and functional analyses of the Nef/NAK interaction will have to await for molecular cloning of this kinase.

Sawai *et al*. (55) have mapped by deletion analysis the region interacting with NAK to the amino acids 45-127 of HIV-1 SF2 Nef which overlaps with the central conserved Nef core domain. However, although SIV mac239 Nef bound equally well to NAK, a reciprocal exchange between SF2 and mac239 Nef proteins of an approximately 60 aa fragment within this conserved core region destroyed the capacity of both proteins to bind to NAK, suggesting that this property is not determined by a simple linear peptide motif (55). In addition to this conserved core region, coprecipitation of NAK with Nef requires the tethering of Nef to the plasma membrane via a functional aminoterminal myristoylation site in Nef, or by fusion to a heterologous membrane-anchoring domain. Within the core domain of Nef Sawai and colleagues identified the second residue (Arg107 in SIVmac239 Nef) of the conserved di-arginine (RR) motif as being critical for NAK association (55). 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 another Nef-binding protein (49). Reversions of mutations disrupting these arginine residues occur in SIV infected macaques before disease progression takes place (56), implying pathogenic importance for the RR motif, although the underlying selection pressures could, of course, also be unrelated to NAK binding.

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, as well as the ability to increase HIV particle infectivity and to block TCR-initiated signal transduction (28, 29). However, since mutation of the second exposed arginine alone is sufficient to completely disrupt NAK association (28, 29), it is a pity that the published data on HIV infectivity both from macaques (56) as well as from cell culture (29) is based on the use of a double RR-to-LL mutant which could potentially be more pleiotropic in its effects, and at least in some studies has been reported to result in an unstable protein (28). On a critical note, it may also be relevant to point out that certain Nef alleles from apparently fully pathogenic strains of HIV-1, such as NL4-3, may associate poorly with NAK even in the absence of any experimentally introduced mutations (92).

Interestingly, an intact PxxP motif has also been found to be required for coprecipitation of NAK activity with Nef (29). 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, but several possible scenarios can be envisioned. First, unlike PAKs, NAK itself or another protein involved in the NAK/Nef complex might contain an SH3 domain, which could stabilize this complex in a PxxP-dependent manner. Second, like its myristoylation (55), PxxP-mediated protein-protein interactions of Nef could be required for correct subcellular localization of Nef in order to allow the NAK complex to form. Third, because NAK can be readily visualized only through an *in vitro* kinase assay of Nef immunocomplexes, it is possible that the PxxP motif might not be involved in binding of Nef to NAK *per se*, but could rather contribute to the activation of NAK, and thus to its detection due to autophosphorylation. The requirement for the PxxP motif for interaction of HIV Nef with both Src-family tyrosine kinase as well as NAK highlights the functional importance of this conserved motif, but also complicates the conclusions regarding the relative contributions of these two classes of kinase as effectors Nef functions that are lacking in mutants in which the PxxP-motif has been disrupted. Additional mutagenesis studies based on the detailed structural understanding of the Nef/SH3 interaction, and possible molecular cloning of NAK, should help to resolve this issue.

As already discussed, conflicting results regarding the requirement of the Nef PxxP motif in the SIV/macaque model of AIDS have recently been obtained. While reversion of the PxxP binding and ability to associate with NAK was seen in all animals in which high viremia and simian AIDS developed by Luciw and colleagues (P. Luciw, personal communication), an opposite result was reported by Kirchhoff and colleagues (76). They reported, however, that the PxxP-to-AxxA mutated SIVmac239 protein could still bind to Src, suggesting that SIV Nef might have redundant strategies for interacting with the Src family of tyrosine kinases. On the other hand, since reversion of the PxxP motif, and thus binding to NAK, was not commonly observed in these animals, they made the provocative conclusion that the NAK interaction would be altogether dispensable for SIV Nef to promote disease progression. Given the abundance of data suggesting a role for NAK in Nef action, as well as the discussed limitations in the study reported by Kirchhoff and colleagues, further investigations into this issue seem warranted.

3.2.2. Other serine kinases associated with Nef

Smith *et al*. have reported the identification of the 80 kD theta isoform of protein kinase C (PKC) as a protein co-precipitating from Jurkat cell cytosolic lysates with GST-Nef fusion protein (58). This binding was not dependent on calcium, and was increased by phosphatidylserine and diacylglycerol, thus correlating with enzymatic activation of PKC-theta. The interaction site in Nef was not mapped, but through the use of a PKC pseudosubstrate peptide it was shown that this interaction was not mediated via the PKC substrate binding site. The proposed Nef/PKC-theta interaction appears to modulate the cellular activity of PKC, since the normal relocation of PKC-theta, but not of a panel of other tested PKC isoforms, to the particulate cellular fraction upon PMA/PHA stimulation was inhibited in Jurkat cells expressing Nef. The possible role of the Nef/PKC-theta in mediating cellular effects of Nef was not address in the study by Smith *et al*, and remains an interesting area for further investigations.

As already mentioned, a recent report by Baur *et al*. (53) identified a cellular serine kinase in a multiprotein complex involving Nef and the tyrosine kinase Lck. 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+. This kinase phosphorylated both Nef and Lck on serine residues, and could interact with number of different HIV-1 Nef sequences, including SF2 and NL4-3, as well as with SIVmac239 Nef. Baur and co-workers suggested that this interaction is mediated by an amphipathic alpha-helix formed by Nef amino acids 16-22 whose predicted secondary structure is well conserved despite the primary sequence divergence among different Nef alleles in the aminoterminal region. Deletion of the amino acids 16-22 resulted in loss of most of the kinase activity, and an intermediate replication phenotype in the resting PBMC assay, when compared to isogenic wild-type and Nef-minus viruses. The ability of the aa 16-22 deleted Nef to downregulated CD4 was not significantly affected.

In this study, molecular weight of this kinase was not definitively identified, but the kinase was suggested to represent one of the several minor 70-80 kD phosphoprotein bands which were seen in the gels, but missing when in vitro kinase reactions of immunoprecipitates containing the aa 16-22 deleted Nef mutant were analyzed. However, in subsequent investigations by Baur and colleagues this serine kinase has been identified as the delta isoform of PK-C (Sass *et al*., submitted). Through the use expression vectors for various isoforms of PKC and corresponding dominant inhibitory mutants of these, as well as various chemical inhibitors of PKC, they found several lines of evidence supporting the notion that the kinase associating with and phosphorylating the aminoterminus of Nef is indeed PKC-delta, whereas no role for PKC-theta previously implicated by Smith *et al*. (58) could be demonstrated. Interestingly, the studies by Sass *et al*. also indicated a possible involvement for the Nef/PKC-delta interaction in the Nef-induced enhanced HIV-1 replication *in vitro*.

3.3. How similar are HIV and SIV Nef?

As already implied on a few occasions, an increasing number of observations are accumulating to suggest that the Nef proteins encoded by HIV-1 and SIV (and HIV-2) might functionally differ from each other. As suggested by the well-conserved core domain shared by both proteins, many of their functions are probably identical, but on the other hand, the divergent regions in their sequences, in particular the aminoterminal part, where SIV Nef contains a large “insertion” accounting for its higher molecular weight, suggest that they may also posses unique functions or different strategies to perform the same functions (figure 4).

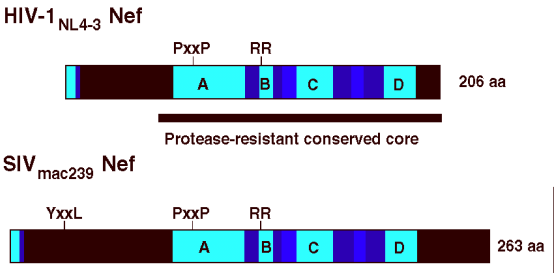

Figure 4. Comparison of the primary stuctures of Nef proteins encoded by HIV-1NL4-3 and SIVmac239. The degree of amino acid conservation is indicated by the intensity of the red colouring, the pale yellow areas thus representing regions with little or no sequence homology between HIV-1 and SIV Nef. The "Nef boxes" A, B, C, and D, as defined by Shugars *et al.* (93), 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 crystallography7(49; Figure 3), and corresponding to the HIV protease cleavage product observed in virions (12, 13) is indicated by the white bar.

It has been shown that the physical requirements (critical amino acids) in the intracellular tail of the CD4 molecule which are necessary for being a subject to downregulation by HIV-1 Nef are different from those required for downregulation by SIV Nef (94). Besides supporting the idea of a direct interaction between CD4 and Nef, this observation also suggest that HIV and SIV Nef proteins bind and downregulate CD4 in a different manner, and that the role of other co-factors in this process might also be different. Recently, a study involving an extensive comparison of the effects of corresponding mutations in the HIV and SIV Nef proteins on their ability to downregulate CD4 and to block TCR-mediated signaling was reported by Skowronski and colleagues (28) Several interesting points were noted. The PxxP motif and other residues implicated in SH3-binding to HIV-1 Nef were found absolutely critical for inhibiting TCR signaling but dispensable for CD4 downregulation, thus supporting and extending previous conclusions (25). However, a similar PxxP-to-AxxA mutation introduced into the SIVmac239 Nef protein had only a minor effect on its ability to block TCR-initiated signal transduction. As suggested above for CD4, it therefore appears that SIV Nef can utilize alternative or redundant strategies for modulating cellular signaling pathways. Given that the effects of Nef on cellular signaling appear to be correlated with its ability to increase viral replication, it is tempting to speculate that such an SH3-binding site (PxxP) independent mechanism of SIV Nef for inhibiting TCR-induced signaling might be related to the observed pathogenicity of SIV carrying a PxxP-to-AxxA-mutated form Nef in some animals (76). Since the PxxP motif is critical for HIV-1 Nef to bind to Src family kinases via their SH3 domains (25, 50), it is of interest to note that the SIVmac239 Nef carrying a PxxP-to-AxxA mutation was still found to be able to associate with Src (76). Thus, the capability of SIV Nef to interact with Src family members via SH3-dependent as well as SH3-independent mechanisms could indeed be an example of the suggested functional redundancy of SIV Nef, as compared to HIV-1 Nef.

An attractive candidate for mediating the SH3-independent association of SIV Nef with Src is a tyrosine-based motif located in the unique region of SIV/HIV-2 Nef proteins. This conserved motif consists of tyrosine and leucine residues arranged in a YxxL configuration, which bears resemblance to the immunoreceptor tyrosine-based activation motif (ITAM) mediating the association of various intracellular components immunoglobulin-family receptor complexes with cellular tyrosine kinases through their phosphotyrosine-binding SH2 domains (for a review, see ref. 95). Interestingly, an Arg-to-Tyr mutation present in the Nef gene of the acutely lethal SIVpbj strain provides it with two tandem YxxL motifs separated by seven amino acids, an arrangement closely resembling a typical high-affinity SH2-binding ITAM (32, 95). The Arg-to-Tyr mutation was shown to account for the unusual properties of SIVpbj Nef, namely its ability to bind Src more efficiently than SIVmac239 Nef, to transform NIH3T3 fibroblasts, and to allow SIV carrying such a Nef allele to replicate in PBMCs without exogenous stimulation of the cultures (32). It is conceivable that the single YxxL sequence observed in other SIV sequences, such as that of SIVmac239 (figure 4), could serve a similar albeit less pronounced function. Thus, SIV Nef may have evolved to posses both SH2 and SH3 domain-based mechanisms to interact with and activate Src family tyrosine kinases, whereas HIV-1 Nef appears to more selectively rely on an SH3-dependent mechanism.

4. CONCLUDING REMARKS

Despite the recent advances in research on Nef, its critical cellular function and molecular mechanism of action in the pathogenesis of AIDS remain unclear. A number of observations suggest that physical and functional interactions with host cell protein kinases are important for Nef function. However, the relevant protein kinases in various target cells of HIV infection still await for definitive identification, posing a major challenge for future investigations.

Molecular cloning of the Nef-associated serine kinase activity, NAK, would seem necessary in order to conclusively address its possible involvement in the cell biology of Nef. The role(s) of different isoforms of PKC as effectors of Nef will also need to be carefully elucidated. On the other hand, the potential role and the downstream molecular events of the Nef/Hck interaction in providing HIV-infected macrophages with a capacity to promote HIV infection alsio requires furtehr experimental attention. Also, the suggestive but contradictory reports on an interaction of Nef with the T cell-specific Src-kinase Lck will have to be followed-up in more detail. If indeed both Hck and Lck turn out to be important, it will be interesting to see if they serve corresponding functions, or whether Nef has evolved to employ them for different purposes in these two cell types.

Although the number of suggested Nef-interacting host cell proteins has rapidly increased, it is likely that not all cellular partners of Nef have yet been identified. In this regard, search for novel high-affinity Nef-binding SH3 domain-containing proteins in non-myeloid cells, such as T lymphocytes, poses one attractive area for further investigations. Besides host proteins involved in signal transduction, other types of novel Nef-interacting proteins are also likely to be discovered, as all pieces in the puzzle regarding the Nef-induced downregulation of CD4 and MHC I are probably not yet at hand.

While the identification of the relevant Nef-binding host cell proteins will undoubtedly accelerate the discovery the critical cellular processes which Nef modulates in order to mediate its pathogenic function,

the reverse argument can also be made. A better idea of the germane Nef-induced changes in cellular physiology would greatly help to decide which ones of the candidate Nef-binding host cell proteins are in fact relevant. In this regard, our current level of understanding leaves a lot of room for improvement. For example, it is still unclear if the true role of Nef is to inhibit or enhance the intracellular signaling mediated through the T cell receptor complex, or whether depending on circumstances both might occur, or even, if both of these effects merely represent different artifacts of ectopic Nef overexpression. Given the likely role of Nef as a regulator of cellular signaling pathways, it is possible that the common practice of using established cell lines to study the effects of Nef on cellular physiology may not have been optimal, and study systems which would better mimick Nef expression during natural HIV infection may have to be considered more carefully in the future.

SIV infection of rhesus macaques has been regarded as the ultimate test for the functionality of various Nef alleles, and it is indeed the model in which the requirement for Nef in the pathogenesis of AIDS was first demonstrated. 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 which (besides being important for any infectious disease) might be relevant to appropriately reveal the *in vivo* function of Nef. The possibility to examine the reversion of mutations in Nef which inhibit the replicative potential of the virus provides another valuable experimental tool in this model. However, besides the broader question of the overall similarity of simian AIDS in SIV-infected macaques compared to AIDS in HIV-infected humans (target cell populations, immunopathogenesis, etc.), there are indications, as discussed in the previous chapter, suggesting that SIV Nef might differ from HIV-1 Nef in certain functional aspects. Such differences, even if subtle, could significantly complicate the use of this model for testing of hypotheses based on studying HIV-1 Nef.

The lack of complete functional correspondence of HIV-1 and SIV Nef proteins is also suggested by the efforts to create a pathogenic SIV strain in which the Nef gene has been transplanted from HIV-1. While such exercises involving exchanges of much larger fragments from other regions of these genomes have been quite successful, substitution of SIV Nef with HIV-1 Nef has proved difficult, and positive results on this have been obtained only recently (P. Luciw, personal communication). The development of such chimeric SHIV viruses represents a significant advance, and should help to reduce the possible problems arising from dissimilarity between HIV-1 and SIV Nef proteins. It should be noted, however, that accumulation of approximately half a dozen amino acid changes in certain positions of the transplanted HIV-1 Nef are required for adaptation of these SHIVs to become pathogenic in macaques.

Besides providing an improved model for *in vivo* testing of the functional importance of specific mutations introduced into the HIV-1 Nef, such SHIV viruses might prove valuable in evaluating possible anti-HIV therapeutic agents targeted against Nef. It is probably realistic to deem that in order to ensure continuous agreeable progress in antiretroviral therapy against AIDS, new drugs against the currently targeted as well as other HIV proteins need to be developed. The demonstrated role of Nef in the pathogenesis of AIDS, together with the improved understanding about the molecular details of HIV-1 Nef structure and function make Nef an attractive target for anti-HIV drug development. The necessity for HIV-1 Nef protein, in order to remain functional, to preserve its architecture in critical regions which interact with host cell proteins, such as the Nef SH3-binding surface, present feasible submolecular targets within the Nef protein, and suggest that the therapeutic effects of drugs aimed at such sites might be less prone to be overcome by mutated drug resistant viruses than is the case with the current antiretroviral therapies.

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