Nef and PAK: virulence factor and cellular accomplice

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The Nef protein is important in HIV replication and in AIDS pathogenesis. The finding that Nef activates a member of the PAK family of protein kinases provides new insights into the mechanisms of action of this critical virulence factor.

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The genomic complexity of lentiviruses sets them apart from other retroviruses. Oncoretroviruses contain only the prototypic *gag*, *pol* and *env* genes, but the human and simian immunodeficiency viruses (HIV and SIV, respectively) have six to seven additional reading frames. Some of these encode proteins that are not essential for viral replication *in vitro*, and are therefore called accessory proteins. This appellation is misleading, because the accessory proteins of primate lentiviruses are essential virulence factors *in vivo*, and are therefore valuable targets for the development of antiviral therapies. One of these proteins, Nef, was revealed to be essential for high levels of viral replication and for disease progression, at least in adult animals, in studies using the SIV/rhesus macaque model of AIDS pathogenesis [1]. More recently, strains of HIV-1 that are defective in Nef function have been suggested to be important in some cases of patients who have been infected with HIV-1 for long periods but have not progressed to disease [2,3]. Thus, drugs that block the activity of Nef might well be useful in the treatment of HIV-1 infection.

Effects of Nef on the virus and its host cell

Nef is a small cytoplasmic protein that associates with the plasma membrane and the cytoskeleton through its myristoylated amino terminus. It is produced from the earliest stage of viral gene expression, affecting both the kinetics of virus replication and the biology of the host cell. First, Nef stimulates the infectivity of HIV particles, apparently by increasing the efficiency of reverse transcription. Although it is possible that this might be a direct effect, because some Nef is found in viral particles, recent data suggest that Nef enhances the serine phosphorylation of the matrix protein, which is a component of the reverse transcription complex, and its effects might thus be indirect (S. Swingler, P. Gallay, J. Song and D.T., unpublished observations). Nef also induces the down-regulation of CD4, one of the cellular receptors for HIV-1, and of major histocompatibility complex (MHC) class I molecules, although this down-regulation is less profound. Nef causes accelerated internalization of these cell-surface molecules by acting as a physical connector to the endocytic apparatus [4]. Finally, Nef alters cellular activation pathways, although there is controversy about how it does so. Defective activation patterns have been reported in human T lymphoid cell lines, or in murine fibroblastic cell lines that stably express Nef [5–7], but elevated signaling has been observed in other systems, including thymocytes from *nef* transgenic mice [8]. In one study, Nef activated T-cell signaling when bound to the plasma membrane, but inhibited it when retained in the cytosol [9]. To explain this paradox it was proposed that Nef sequesters a putative signaling molecule that is active only when localized at the plasma membrane.

Nef and protein kinases

Nef has no known catalytic activity, so it is likely to exert its effects via cellular proteins, particularly those involved in signal transduction. Nef has so far been shown to associate with two kinds of protein kinases. First, it binds to some members of the Src family of tyrosine kinases. This interaction is mediated through the recognition of a conserved (PxxP) repeat in Nef by the SH3 domain of these enzymes [10,11]. Although the exact nature of the Src-related kinase that binds to Nef within the context of infected cells is as yet unknown, functional evidence suggests that it participates in stimulating viral replication [10]. Second, a cellular serine/threonine kinase activity can be immunoprecipitated with Nef proteins from HIV and SIV [12]. *In vitro*, this activity phosphorylates substrates of 62 kDa and 72 kDa that are also bound to Nef. The larger protein, p72, is detected in T lymphoid cells but not in COS cells, suggesting that it might be a lymphocyte-specific substrate for the Nef-associated kinase. Nef only binds to the protein serine kinase activity when it is associated with the plasma membrane, and binding requires the presence of two arginine residues within the central region of the viral protein [13].

Until recently, the identity and functional importance of the Nef-associated serine kinase were unknown. Three recent papers have shed some light on this question [14–16]. Nunn and Marsh [14] first showed that the Nef-associated kinase can be immunoprecipitated using antibodies to a member of the family of p21-activated kinases (PAKs). PAKs are highly conserved, ubiquitous protein serine kinases that are activated after associating with the p21 Rho-like GTP-binding proteins Rac1 and Cdc42Hs, which themselves are functionally linked to various growth factor and mitogen receptors. PAKs are the most upstream enzymes of a cascade that leads to the activation of mitogen-activated protein kinases (MAPK) such as the

c-jun amino-terminal kinase (JNK), and have been implicated in a variety of processes including cellular activation and actin polymerization [17–20]. In T lymphocytes, one member of the PAK family could participate in stimulating the activity of inducible Jun-containing transcription factors such as NF-AT and NF-IL2, and thus in the response to CD3–CD28 costimulation, via a pathway involving the activation of JNK [21].

Nunn and Marsh [14] also found that the Nef-associated serine kinase, like members of the PAK family, could readily use histone H4 as a substrate and was stimulated by overexpression of constitutively activated (i.e. GTP-bound) forms of Rac1 and Cdc42. Taken together with the serological evidence, these data suggest that the Nef-associated kinase may also be part of the PAK family.

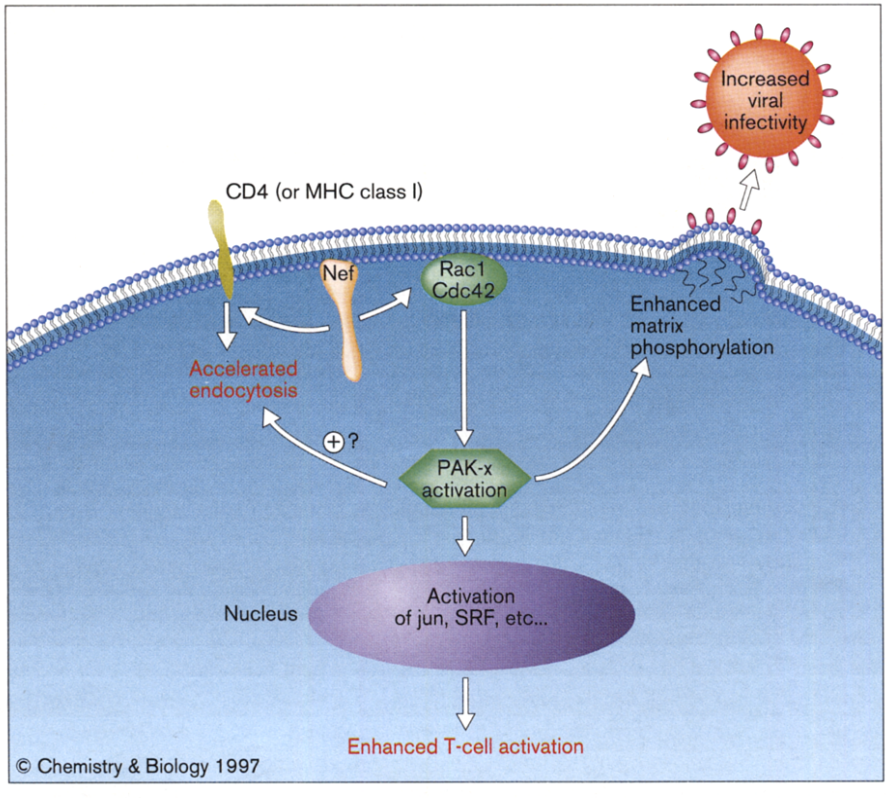
Two further publications [15,16] confirm and extend these data. Sawai *et al.* [15] and Lu *et al.* [16] also showed that antibodies against PAKs could recognize the protein serine kinase activity associated with HIV-1 or SIV Nef in either Nef-expressing or virus-infected cells, and detected 62 kDa and 72 kDa phosphoproteins after performing *in vitro* kinase assays on PAK immunoprecipitates obtained from these cells. Sawai *et al.* [15] further showed, using partial chymotryptic digestion, that the p62 and p72 proteins had different patterns, but that the pattern for each protein was identical whether the material was recovered from SIV Nef immunoprecipitates or from anti-PAK antibody immunoprecipitates [15].

Transient expression assays, *in vitro* transcription and translation experiments and partial proteolytic digests indicate that the Nef-associated kinase is distinct from all known PAKs [14,16]. Expression of a dominant negative form of human PAK65 (hPAK65), containing the GTPase-binding region but lacking the kinase domain of this protein, blocked the activity of the Nef-associated kinase, however. Thus the Nef-associated kinase, like other PAKs, appears to bind to the GTPases Cdc42 and Rac1 [16]. Similarly, dominant-negative forms of Cdc42 and Rac1 inhibit the Nef-associated kinase [16].

Importance of the Nef–PAK association

The functional significance of the association between Nef and members of the PAK family of kinases is not yet clearly established, but both tissue culture experiments and studies performed in the SIV/rhesus macaque model provide evidence for its physiological relevance (Fig. 1). We recently found that an HIV-1 variant expressing a Nef mutant protein that was unable to recruit the protein serine kinase activity had lower levels of matrix serine phosphorylation than wild-type HIV-1. These results may provide the link between Nef, its associated kinase, and

Figure 1



Proposed model for the Nef–PAK connection. The three known phenotypic effects of Nef are indicated in red. Nef-induced activation of a PAK-related protein serine kinase (PAK-x) could result in activating a number of transcription factors associated with T-cell activation. In parallel, PAK could promote the infectivity of virions by stimulating the phosphorylation of the matrix protein, one of the virus major structural components. Finally, the GTPases Rho and Rac have been shown to regulate receptor-mediated endocytosis [23]. It is unclear whether Nef affects this pathway, however, as the only molecules found to be down-regulated by Nef are CD4 and, to a lesser extent, MHC class I.

the stimulation of HIV-1 proviral DNA synthesis (S. Swingler, P. Gallay, J. Song and D.T., unpublished observations). The same mutant Nef allele could still down-regulate CD4, albeit less efficiently than wild-type [22]. Lu *et al.* [16] observed that expressing dominant negative forms of hPAK65, or of Rac1 and Cdc42, decreased the efficiency of viral particle production from COS cells that had been transiently transfected with a wild-type, but not a *nef*-deleted, HIV-1 proviral DNA construct. They also noted that the levels of virion release were significantly lower with *nef*-deleted virus than with wild-type virus. This result is quite puzzling, since no other group has previously reported a positive effect of Nef on the efficacy of viral particle production. Finally, they found that Nef activates the serum-response pathway, a known target of small GTPases [19], via the Nef-associated kinase, Cdc42 and Rac1 [16]. The implications of this result, obtained in transiently transfected murine fibroblasts, are not obvious. It is possible that this phenomenon relates to the ability of Nef to alter T-cell activation pathways; perhaps Nef modifies infected cells to the benefit of viral production.

To determine the importance of the Nef-associated serine kinase *in vivo*, Sawai *et al.* [15] used an SIV variant containing a mutation in Nef that abrogates binding to and activation of the Nef-associated protein serine kinase. Rhesus macaques infected with this virus at first had lower viral loads than those infected with wild-type virus, but then rapidly reverted to the normal course of infection, while at the same time the virus reverted to a wild-type Nef genotype. This result shows that the mutated residues, two arginines in the core domain of Nef are crucial for the life cycle of the virus, implying that the Nef-associated kinase is itself essential for viral replication and in AIDS pathogenesis,. One cannot, however, formally exclude the possibility that the mutation might have affected other aspects of Nef function, especially since the two arginines are located within the conserved central region of the protein, and one of them at least may be structurally important [11].

Future directions

Future experiments should further explore the influence of the Nef-associated kinase on the replication of the primate lentiviruses and on AIDS pathogenesis. One important task will be to formally identify which member of the PAK family associates with Nef. The eventual determination of the three-dimensional structure of the complex formed by the viral protein and its cellular accomplice should be an important step towards the discovery of inhibitors, and will considerably assist in the dissection of the pathways affected by this kinase. These studies will not only provide insight into the effects of this enzyme on the viral life-cycle, but will also help us to determine what it normally does in the cell. In this case, as frequently happens, the study of a virus may lead to breakthroughs in the understanding of basic cellular phenomena.

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