Biology of the HIV Nef protein

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The accessory Nef protein is expressed by all primate lentiviruses – HIV-1, HIV-2 and simian immune deficiency virus (SIV). Its expression in the early stages of the viral life cycle ensures two basic attributes of HIV infection. These are T-cell activation and the establishment of a persistent state of infection. Nef has a positive effect on viral infection and replication by promoting the survival of infected cells. Its role in HIV persistence is based largely on the ability of Nef to downmodulate the surface levels of important molecules at the immune synapse. These include major histocompatibility complex-I (MHC I) and (MHC II) present on antigen-presenting cells (APCs) and target cells, and CD4 and CD28 present on helper T cells. In this review we present these biological properties of Nef from a mechanistic point of view, and relate them to the structural attributes and interactions of the Nef protein. A brief outline of the limited studies on Nef from Indian subtype C HIV-1 isolates is also presented.

Key words Apoptosis - HIV-1 - immune synapse - Nef - signaling - T-cell activation

Besides the three prototypical retroviral proteins (Gag, Pol and Env) and two regulatory proteins (Tat, Rev) that are essential for viral replication, HIV (and simian immunodeficiency virus, SIV) also encodes four so-called accessory proteins called Nef, Vif, Vpr and Vpu. While these accessory proteins are dispensable for viral replication in vitro, they perform essential functions during the viral life cycle in the host. In vivo studies or those in primary cell types susceptible to HIV infection have demonstrated that the accessory gene products can dramatically alter the course and severity of viral infection, replication and disease progression. The nef gene is highly conserved in all primate lentiviruses e.g. HIV-1, HIV-2 and SIV and the encoded Nef protein appears to be a virulence factor critical for the development of AIDS 1. It was first identified as an open reading frame (ORF) that partially overlaps with the 3’-long terminal repeat of the HIV-1 and was reported to have a negative effect on viral replication, hence the name ‘negative factor’ or nef 2,4. Since then, extensive studies have shown this to be a misnomer and have characterized multiple cellular pathways that are regulated by expression of the Nef protein.

Here we discuss the biology of Nef and its various effects on the host cell in a bid to promote viral replication and persistence.

Nef as a positive viral factor

Nef is expressed in abundance during the early phase of HIV infection together with Tat and Rev. Its mRNA is estimated to represent three quarters of the early viral mRNA load of the cell 5, 6. Nef induces high viral titres both in cell culture and in vivo. Studies in Rhesus monkeys have clearly demonstrated that an intact nef gene is critical for attaining high virus loads and development of an acquired immunodeficiency syndrome (AIDS)-like
illness in animals infected with the SIV. A similar requirement for the maintenance of high virus loads has been demonstrated for HIV-1 in the SCID-Hu model. In this system, viruses with an intact nef ORF replicate faster, achieve higher titres, and deplete thymocytes better than their nef-deleted counterparts. Moreover, long-term survivors of HIV infection who show lack of disease progression are commonly associated with either a deletion in the nef gene or defective nef alleles. In transgenic mouse models, Nef expression in CD4 positive cells caused an AIDS-like disease. Of note, the Nef protein of SIV and HIV are functionally interchangeable.

Nef was originally characterized as a negative regulator of HIV infection and was thus named as 'negative factor'. In earlier studies, it was found that nef-defective viruses replicate slightly faster than the wild type viruses in CD4+ cell lines. In addition, it was shown that Nef could downregulate HIV-1 LTR activity. However, these initial findings were later refuted by other groups. In most cases, Nef either has no effect or a positive effect on viral replication in vitro. Further, it has also been shown that Nef does not inhibit transcription of the HIV-1 LTR in a variety of cell types.

The Nef-mediated enhancement of viral replication is a system-dependent process. Nef generally has no effect on viral replication in activated peripheral blood mononuclear cells (PBMC), activated CD4+ T cells and mature dendritic cell-T cell co-cultures. On the other hand, a significant role for Nef in viral replication has been found in post-infection-stimulated PBMCs or lymphoid cultures, immature dendritic cell-T cell co-cultures and in the ex vivo tonsil culture system.

Effect of Nef on virion infectivity and replication

The nef gene of HIV is critical for pathogenesis and development of AIDS in humans as well as in animal models. Using the Hela-CD4-LTR-β-galactosidase indicator cell line, nef+ HIV-1 was found to productively infect 5 to 20-fold more cells than equal amounts of nef-defective HIV-1. The infectivity of nef defective HIV-1 can be rescued by expressing Nef in trans in the virus-producing cell. The replication rates of nef+ and nef-defective HIV-1 clones in activated primary blood lymphocytes (PBL) indicated that Nef directly promotes HIV-1 replication by enhancing the infectivity of virions. Viruses produced from proviral DNAs mutated in nef are 4 to 40 times less infectious than the wild type virus in single-round infection assays. The Nef effect is dependent on the association of this protein with the plasma membrane and is determined at the stage of virus particle formation.

The inclusion of Nef in the virion may facilitate the incorporation of Nef-associated cellular kinases that phosphorylate various substrates, including the viral matrix protein, important for the production of fully infectious viral particles. Productive HIV-1 infection is also regulated by the ability of Nef to induce the release of a lymphocyte-stimulating factor by macrophages. This leads to an environment in which Nef promotes viral replication in the host by increasing the pool of substrate lymphocytes without additional stimuli. The effects of Nef on lymphocyte signaling also involve transcription factors that are induced in response to signaling from the T cell receptor (TCR). Manninen et al. described a novel effect of Nef on lymphocyte signaling mediated independently of the TCR, which results in induction of nuclear factor of activated T cells (NFAT), a transcription factor that plays a central role in co-ordinating T cell activation. The effect of Nef on T cells is mediated by activation of the calcium/calcineurin pathway and was synergistic with the Ras pathway in inducing NFAT-dependent gene expression. Ectopic expression of NFAT in resting CD4+ T lymphocytes induced a permissive state, which despite the lack of evidence of T cell activating effects of NFAT overexpression, supported HIV replication in these cells in the absence of further stimulation. HIV replication in these cells is supported...
by the overexpression of NFAT target genes IL-2 and FasL, in the absence of further stimulation. The NFAT protein has also been shown to activate HIV-1 LTR-directed transcription by interacting with an unusual binding site that overlaps with the NF-kB-responsive element. The Nef-mediated superinduction of IL-2 is a reflection of activation of both NFAT and NF-kB sites; thus this mechanism promotes viral replication and spread.

The replication of HIV-1 in vitro is restricted to dividing (activated) T cells and studies detailed above explain how Nef enhances viral replication by T cell activation. However, this first requires expression of Nef in a resting T cell that is refractory to infection. One mechanism put forth is the expression of Nef from unintegrated viral DNA. Swingler et al. recently proposed an alternative pathway in which Nef intersects the CD40 ligand-signaling pathway in macrophages, the first cell type to be infected by HIV. Physiological stimulation of CD40 as also Nef expression in macrophages promotes the release of soluble CD23 (sCD23) and soluble intercellular cell adhesion molecule (sICAM). These in turn upregulate the expression of costimulatory receptors CD22 and CD58 (by sCD23) and CD80 (by sICAM) on B lymphocytes, leading to increased interaction with their corresponding ligands on T lymphocytes. This causes activation and proliferation of T lymphocytes, making them susceptible for HIV infection.

Cell surface expression of critical proteins

The HIV and SIV Nef proteins have been shown to direct the downmodulation of several critical cell surface proteins that are a part of the immune synapse. This is a major viral strategy towards creating a persistent state of infection. The effects and their mechanisms are detailed below:

CD4 downmodulation: The CD4 protein is a 55 kDa type I integral cell surface glycoprotein and a component of the T cell receptor on MHC class II-restricted cells such as helper/inducer T-lymphocytes and cells of the macrophage/monocyte lineage and serves as the primary cellular receptor for HIV and SIV. The most extensively studied function of Nef is its ability to dramatically reduce the steady state levels of CD4 on the cell surface. Almost all Nef isolates downmodulate cell surface CD4 in all mammalian cell types tested, and under almost all experimental conditions. In transgenic mice expressing HIV-1 Nef, a significant decrease in CD4 expression on the surface of immature double-positive thymocytes is associated with compromised positive selection of CD4+ lymphocytes. One of the major benefits of Nef-induced CD4 downregulation could be the enhancement of viral particle release by preventing the sequestration of viral envelope by the CD4 receptor or its influence on the assembly and entry of viral particles. The downmodulation of surface CD4 would also prevent superinfection, an event that would lead to premature death of the host cell.

The Env and Vpu proteins of HIV-1 also downregulate the CD4 receptor. However, in contrast to these two proteins that function at intracellular sites, Nef acts on CD4 molecules that have already reached the cell surface. Nef-induced CD4 downregulation involves accelerating endocytosis of CD4 followed by its degradation through the endo-lysosomal pathway. It has no detectable effect on either CD4 synthesis or transport through the exocytotic machinery. That Nef reduces cell surface CD4 through the endo-lysosomal pathway is supported by the observation that Nef-expressing cells have more numbers of CD4 containing clathrin–coated pits (CCPs). Further, lysosomotropic agents block Nef-mediated CD4 downregulation.

For endocytosis, transmembrane receptors like CD4 commonly contain specific motifs within their cytoplasmic tails that serve as a recognition motif for CCP and that in turn internalize the receptor. The CD4 protein has four hydrophobic amino acids including two leucine residues in the membrane proximal region of its cytoplasmic tail that are necessary for its endocytosis. This site is usually masked by binding of the Src kinase family T-cell-specific protein kinase, p56 Lck that prevents CD4 internalization mediated by binding to the adaptor proteins (AP) in the clathrin complex. Nef appears to bypass the normal routes of CD4 downregulation, which involves phosphorylation of Ser408 in its cytoplasmic tail, by directly binding the dileucine
motif in the CD4 cytoplasmic region. The residues in Nef involved in contacting CD4 are WL (57-58) clustered in a proximal flexible loop, and residues G95, G96, L97, R106 and L110 present in the Nef core domain. The Asp204 residue in Nef is also critical for CD4 binding as its mutation to lysine (D204K mutant) completely abolishes binding to CD4 and its rapid internalization. Additionally, Nef itself contains a dileucine motif, which recruits the clathrin complex AP-2 subunits via its µ2 chain. Mutations of the critical Tyr28 and Tyr39 residues in Nef prevent this interaction, but the D204K mutant still binds to µ2. Thus, Nef acts as a bridge between CD4 and µ2 by displacing p56Lck and its phosphorylation trigger.

There are four different classes of adaptor proteins (AP) found in the cell. Of these, AP1 and AP2 are components of clathrin coats associated with the trans-golgi network (TGN)/endosomes and the plasma membrane; AP-3 exists in both clathrin and nonclathrin coats and is localized to endosomes, while AP-4 is a part of the non-clathrin coat associated with the TGN.

A recent report using the yeast three-hybrid method showed that Nef interacts with AP-1 and AP-3, but not AP-2 and AP-4. Stronger interactions have also been observed between Nef and the µ subunits of AP-1 and AP-3, which involves trafficking between TGN and the endo-lysosomal compartment. These interactions, including results with CD4-Nef chimeras, suggest that Nef might direct CD4 from the TGN directly to endosomes without targeting it to the plasma membrane. The adaptor AP-2 does not interact with Nef and is not a direct target for regulation by HIV-1. However, another protein such as the regulatory µ2 subunit of vacuolar ATPase may be responsible for the Nef-induced removal of CD4 from the cell surface. The LL165/166 and His194 residues of Nef are critical for CD28 downmodulation. The benefit of CD28 surface downmodulation to the virus is not clear. It has been hypothesized that by downmodulating CD28, the virus can block T-cell

Another region of Nef, an acidic dipeptide motif, EE155/156, which was earlier proposed to interact with β-COP (a part of the COP1 coatamer), is important for CD4 downmodulation and was proposed to target the Nef-CD4 complex from early to late endosomes and lysosomes (Fig. 1). The primary isolate Nef233 has an EK motif at this position (instead of EE), but is fully functional in CD4 downmodulation. Thus, Nef mediated β-COP coatamer recruitment is most likely indirect and depends on a critical cofactor. In recent work, Faure et al. have identified the ARF1 GTPase to be this cofactor responsible for formation of the transport machinery via Nef binding (Fig. 1).

Nef has also been shown to bind to the novel human thioesterase II protein and this interaction is important for CD4 downmodulation. However, the Nef protein of the HIV-1 SF2 isolate does not bind to thioesterase but still downregulates CD4. One of the Nef residues critical for thioesterase binding, D123, was also critical for Nef dimerization and was found to be critical for Nef mediated downregulation of CD4 and major histocompatibility complex I (MHC I). Besides dimerization, N-terminal myristoylation of Nef leading to its membrane localization is also critical for Nef-mediated downmodulation of CD4 as well as the other surface markers.

**CD28 downmodulation:** The functional outcome of TCR engagement is normally decided by the presence or absence of co-stimulatory signals delivered via accessory molecules such as CD28. Ligation of either TCR or CD28 alone induces minimal levels of T cell activation, which leads to a state of anergy. In addition to surface downmodulation of CD4, Nef also accelerates internalization of the CD28 co-stimulatory molecule, which is necessary for maximal T-cell activation. Genetic and functional studies suggest that Nef-mediated endocytosis of CD28 is AP-2 dependent and is mediated by a direct interaction between Nef, AP-2 and CD28. This activity of Nef is a conserved phenomenon in multiple isolates of HIV-1 and SIV. The LL165/166 and His194 residues of Nef are critical for CD28 downmodulation.

The benefit of CD28 surface downmodulation to the virus is not clear. It has been hypothesized that by downmodulating CD28, the virus can block T-cell
activation. The interactions between CD28 and B7 molecules and between TCR/CD4 and MHC II molecules are thought to make critical contributions to the stability and maintenance of a tight and prolonged interaction between T cells and antigen presenting cells (APCs). The Nef-mediated downmodulation of CD28, in addition to CD4, probably limits the strong interaction of Nef-expressing T-cells with APCs. This would enhance subsequent movement of infected T-cells into the circulation and to other APCs to accelerate the spread of the virus.

Alternatively, by downmodulating CD28, Nef can minimize signaling upon engagement of the infected T-cell with an APC or interfere with normal TCR-initiated signaling. This effect may be necessary to prevent activation-induced apoptosis of already activated and infected cells by further stimulation through CD3. As Nef activates certain downstream effectors of signaling pathways such as NFAT-1 and IL-2, it can replace the normal signaling cascades that govern antigen-specific T-cell activation and signaling. It is plausible that the infected cell can still

Fig. 1. Nef-induced downmodulation of CD4 and MHC molecules. Models are presented based on available data on the downmodulation of surface-expressed CD4, MHC I and MHC II. For CD4 downmodulation, Nef connects the cytoplasmic tail of CD4 with adaptor protein 2 (AP2) and the V1H vacuolar ATPase, triggering rapid CD4 endocytosis (1). In the early endosome, Nef interacts with COP1 coamperomers through the ADP ribosylation factor 1 (ARF1) and targets CD4 for lysosomal degradation (2). Nef is targeted to the trans-Golgi network (TGN) (3) where it acquires the ability to activate PI3K (4). The resulting formation of phosphatidylinositol-3,4,5-triphosphate (PtdInsP) recruits the guanosine exchange factor ARNO to the plasma membrane leading to the activation of ARF6 (5). This accelerates the endocytosis of MHC class I molecules into an ARF6-dependent compartment (6) and subsequently through a PACS-1/AP1-dependent step into the TGN (7). The MHC class II molecules possibly bind Nef at the plasma membrane and are relocated to the early endosome (8) enroute to their lysosomal degradation (9). The activation of PI3K and ARF6 are shown by a change in color from light to dark. Steps for which mechanistic details are not fully available are shown as dash arrows. (concept of figure adapted from Fig.5 of B.M. Peterlin and D. Trono. Nat Rev Immunol 2003; 3: 97-107).
remain activated yet protected from improper signaling through CD28 downmodulation, thus disconnecting T-cell activation from antigen presentation\textsuperscript{74, 75}.

\textbf{MHC class I downmodulation}: Adaptive immune responses require recognition of infected cells prior to their elimination by cytotoxic T lymphocytes (CTLs). This requires viral peptide presentation on the surface of infected cells by MHC I molecules\textsuperscript{76-78}. To counteract this host defense mechanism, several persistent viruses such as herpesviruses, poxviruses and retroviruses, have developed strategies that target the assembly and trafficking of newly synthesized MHC I molecules in infected cells\textsuperscript{78}. This alters the MHC I presentation of viral peptides and promotes the escape of virus-infected cells from CTL surveillance. Notably, the Nef protein expressed in the early stages of HIV infection carries out this function. Following infection by HIV/SIV, Nef downmodulates surface MHC I and this function is critical for the establishment of infection and subsequent development of AIDS. The downmodulation of MHC I protects HIV-infected cells from CTL-mediated killing and provides a selective advantage for viral persistence and replication \textit{in vivo}\textsuperscript{76}. Nef selectively downregulates HLA-A and HLA-B, which present antigens to CTLs but not HLA-C and HLA-E which helps protect cells from lysis by natural killer (NK) cells\textsuperscript{79-82}. A cytoplasmic tyrosine residue unique to the HLA-A and HLA-B proteins confers this specificity. However, it should be noted that Nef does not protect completely from immune surveillance, as there is a strong CTL response to HIV infection.

Heterotrimeric functional MHC class I complexes consists of the MHC I heavy chain noncovalently attached with β2 microglobulin (β2m) and loaded with an 8-11 amino acid peptide. The assembly of these two components and the loading of antigenic peptides that are generated by the proteosome occur in the endoplasmic reticulum (ER). Many DNA viruses such as adeno, herpes and poxviruses interfere with peptide-MHC I presentation at the level of assembly of this complex within the ER\textsuperscript{83}. Nef does not affect these early processes, but speeds up the internalization of cell surface MHC I molecules\textsuperscript{83-85}.

Mutagenesis studies reveal that Nef-mediated downregulation of CD4 and MHC I molecules by endocytic trafficking occurs via distinct pathways (Fig. 1). In the presence of Nef, the synthesis of MHC I molecule is unaltered and these molecules transport normally through the ER/Golgi route. However, MHC I molecules are rapidly internalized from the cell surface through the endosomes, transported back to accumulate in the TGN, and misdirected into post-Golgi clathrin-containing vesicles in Nef expressing cells\textsuperscript{74, 75}. Inhibitors of phosphatidylinositol 3-kinase (PI3K) block this effect\textsuperscript{86}. The phosphofurin acidic cluster-sorting signal 1 (PACS1) protein is required for Nef-mediated downregulation of MHC I to the TGN by binding to the Nef acidic cluster EEEE (amino acids 62-65)\textsuperscript{87}. While PI3K is important for TGN-derived clathrin-coated vesicles, PACS1 is required for early endosome to Golgi trafficking, as in the case of furin-mannose-6 phosphate receptor (M6PR) transport\textsuperscript{88}. The interaction of Nef with MHC I is transient\textsuperscript{89} and two additional Nef sorting motifs are critically important for MHC I downregulation. These include the Met20 residue within the amphipathic helix and the PXXP motif-containing SH3-domain binding site\textsuperscript{86, 90}.

Dominant-negative proteins and chemical inhibitors have been used to explain the sequential roles of the three Nef sorting motifs\textsuperscript{86}. MHC I downregulation is initiated by the binding of the Nef acidic cluster (EEE; 62-65) to PACS-1/AP-1. This is a crucial step to the subsequent PXXP (residues 72-75) mediated ADP ribosylation factor 6 (ARF6) activation\textsuperscript{86, 91}. The ARF6 protein connects vesicles trafficking with actin cytoskeletal rearrangement, and this endocytosis and recycling of proteins through the ARF6-dependent endosomal compartment is regulated by the GTP/GDP bound state of ARF6. Nef activates the ARF6 GTPase through PI3K and the guanosine-exchange factor (GEF) ARNO, thereby triggering the clathrin-independent transport of MHC I molecule from the cell surface to the TGN through an ARF6 compartment\textsuperscript{86}. It has also been proposed that the apparent ARF6 dependence of Nef-mediated MHC I downmodulation could be due to nonspecific perturbations in membrane trafficking\textsuperscript{92, 93}. The steps in MHC I downmodulation by Nef are illustrated in Fig.1.
MHC Class II downmodulation: The MHC II protein is expressed in all APCs and present antigenic peptides to CD4+ T cells⁹⁴. Thus, it is present on all cell types that are infected by HIV. Further, human macrophages accumulate HIV-1 particles in MHC II compartments⁹⁵ and MHC II can be incorporated in the virion⁹⁶. In chronically infected monocytes, MHC II antigen presentation is hampered⁹⁷, and HIV-1 Nef is reported to affect the surface localization of MHC II proteins, reducing presentation of exogenous peptides to CD4+ cells⁹⁸-¹⁰⁰.

The presentation of antigenic peptides in the context of MHC II is central to the adaptive immune system whereby peptides generated from exogenous sources are presented to T-helper cells.

The Nef protein disrupts MHC II antigen presentation by two distinct mechanisms. These include downregulating surface expression of mature MHC II and upregulating surface expression of MHC II associated invariant chain (Ii, CD74). Higher amounts of Nef expression are required for mature MHC II downmodulation compared to upregulation of Ii. Nef-alleles from primary HIV-1 cause surface upregulation of Ii, a function that is genetically separable from MHC II surface downmodulation¹⁰⁰. For example, the acidic domain of Nef is involved in downregulation of MHC II but is dispensable for Ii upregulation. Mutations in the C-proximal flexible loop consistently abolish the ability of Nef to modulate Ii surface expression but had little effect on downregulation of MHC II. The dileucine motif LL165/166 and the acidic motif EDE174-176 are critical for Nef-induced downregulation of CD4 and upregulation of Ii. Mutations in Pro75 and Pro78 block MHC I and MHC II downmodulation but have no effect on Ii upregulation, suggesting that an intact SH3-binding PxxP motif is not required for Ii upregulation but is required for MHC II modulation⁹⁸-¹⁰⁰. These results illustrate the mechanistic details of MHC II downmodulation by Nef and emphasize the importance of this function in viral immune evasion through inhibition of virus-specific T cell help.

T-cell receptor and cellular signaling pathways

Stimulation of the TCR on mature T cells induces a series of biochemical events, which eventually result in the induction of gene expression in the activated T cell. The initiating signals are activated protein tyrosine kinases, including members of the Src, Syk/Zap-70 and Tec families. These kinases phosphorylate their target substrates resulting in the modification or metabolism of membrane phospholipids mediated by enzymes such as phosphatidylinositol 3-kinase (PI3K), phopholipase C (PLC) and calcium homeostasis. This leads to the production of second messengers such as diacylglycerol and inositol triphosphate, and the activation of small GTP-binding proteins, such as p21Ras. The ultimate outcome of this complex intracellular signaling is the activation of transcription factors leading to gene expression¹⁰¹.

The culture of macaque peripheral blood lymphocytes⁷ and a herpes virus samiri-infected macaque T cell line require the addition of IL-2. Unlike lymphocytes, this cell line becomes IL-2 independent when infected by nef+ SIV but not by nef-defective SIV¹⁰², suggesting that Nef can play a direct and positive role in T cell activation. Microarray analyses have also shown that the total expression profiles of TCR-activated T cells and Nef expressing cells are 97 per cent similar¹⁰³. Nef might initiate the activation process by myristoylation-dependent translocation to the plasma membrane and its interaction with a variety of signaling proteins¹⁰⁴. Many of these signaling proteins, like Lck or linker for activation of T cells (LAT), are at least partially present in glycolipid-enriched microdomains (lipid rafts) where Nef is also found¹⁰⁵. Aggregation of lipid rafts initiates T cell signaling, similar to the manner of receptor stimulation by ligands. By binding to molecules of different compartments, including rafts and possibly by forming oligomers, Nef may function as an intracellular cross-linker or adaptor.

The Nef protein lacks any catalytic activity and acts by influencing kinases or other signaling pathways within the host cell¹⁰⁶. The strong conservation of proline-rich motifs among various Nef alleles and SH3-dependent activation of Src family tyrosine kinases may be a general property of primate lentiviruses. The interaction of Nef with the Hck SH3 domain has been shown to be the tightest SH3 domain-ligand interaction so far reported, with a dissociation constant K_D of 0.2 µM¹⁰⁷. Stimulation of the Hck tyrosine kinase activity by Nef depicts a
particularly extreme phenotype resulting in malignant transformation of Rat2 fibroblasts\textsuperscript{108}. Hck is rapidly induced following macrophage activation and has been implicated in multiple signaling events including phagocytosis, Fc receptor signal transduction, integrin signaling, and tumour necrosis factor release\textsuperscript{109}.

Two phosphoproteins with molecular weights of 62,000 and 72,000, called Nef-associated kinases (NAKs), were found to co-immunoprecipitate with Nef\textsuperscript{110}. One of these proteins is the p21-activated kinase 2 (PAK2)\textsuperscript{111}, that has direct effects on cytoskeletal morphology and apoptotic signaling. Nef may 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 steps such as transcription, reverse transcription or budding.

The enzyme PI3K consists of a regulatory and a catalytic subunit. Following the generation of phosphatidylinositol (PtdIns)-3,4,5-Pi, a variety of proteins are recruited to different membranes via their lipid binding domains. Among them are the exchange factors for small GTPases of the Rho, ARF family and the PDK-1 families\textsuperscript{112}. The relocalization of these proteins initiates a broad range of cellular effects such as proliferation, membrane ruffling, prevention of apoptosis, and certain vesicular transport functions. Nef binds the regulatory p85\textsubscript{α} subunit of PI3K. It is possible that Nef assembles PI3K, Vav, and PAK into a signaling complex, which is required for increased viral production in a Nef-dependent manner\textsuperscript{113}.

The Nef protein has also been reported to bind the c-Raf 1 kinase, which plays an important role in co-ordinating the Ras-Raf-MAPK pathway\textsuperscript{114}. This particular binding is dependent on a conserved DDPxxE\textsubscript{174} motif in Nef, which resembles an acidic consensus Raf-binding motif previously characterized in p21\textsuperscript{Ras}. Nef is also reported to bind to the 80-kDa theta isofrom of protein kinase C (PKC\textsubscript{θ}); this interaction is reported to modulate PKC activity, since its normal relocation to the particulate cellular fraction was inhibited in Jurkat cells expressing Nef\textsuperscript{115}. Nef also associates with the receptor for activated C kinase 1 (Rac1), a known intracellular receptor for PKC\textsubscript{s}\textsuperscript{116}. Rac1 is in equilibrium between a cytosolic and membrane bound state, partially colocalizes with Nef, and enhances its \textit{in vitro} phosphorylation by PKC\textsubscript{s}\textsuperscript{117}.

The expression of Nef in T cells unleashes a series of signaling events that are similar to those that occur following T-cell activation through the TCR and co-stimulatory pathways. This results from the ability of myristoylated Nef protein to localize at the plasma membrane and assemble multiprotein signaling complexes through its various interacting domains.

### Nef and apoptosis

HIV induces apoptosis in both infected and uninfected immune effector cells\textsuperscript{118}. Nef induces the expression of both Fas (CD95) and the Fas ligand (CD95L) in infected cells\textsuperscript{119, 120}, with CD95L aiding immune evasion by inducing the apoptosis of HIV-specific cytotoxic T-cells (CTLs)\textsuperscript{119}. The apoptosis signal regulating kinase 1 (ASK1) is a key signaling intermediate in the Fas and TNF-α death signaling pathways and was reported to bind Nef\textsuperscript{121}. This association is reported to result in the inhibition of ASK1 kinase activity (Fig. 2) and the downstream induction of c-Jun N-terminal kinase (JNK) and apoptosis\textsuperscript{122}. The binding of Nef to ASK1 is lost on disruption of its N-terminal myristoylation site as well as by a R106A mutation, which is implicated in the binding of a p21–activated kinase (PAK) family protein\textsuperscript{123}.

The association of Nef with ASK1 reveals a mechanism by which HIV-1 Nef can alter the intracellular milieu of virally infected host cells by enhancing their resistance to Fas and TNF-α mediated apoptosis (Fig. 2). The Nef protein also represses death signaling by Bad, a pro-apoptotic member of the Bcl-2 protein family whose expression is induced by HIV, and that triggers apoptosis at the level of mitochondria\textsuperscript{123}. The Nef-mediated activation of PI3K and PAK results in the phosphorylation of Bad resulting in release of the anti-apoptotic Bcl-X\textsubscript{L} protein from a Bad/Bcl-X\textsubscript{L} complex and enhancement of cell survival and virus production\textsuperscript{124}. Through its N-terminus (residues 1-57) Nef also interacts with the p53 tumour suppressor protein. This interaction results in the destabilization of p53, thereby decreasing its pro-apoptotic, transcriptional and DNA-binding
activities, and protecting HIV-1 infected cells from p53-mediated apoptosis. Thus Nef protects the infected cell through two mechanisms. It blocks external death signals coming from CTLs or auto-reactive FasL through the inhibition of Ask1. Internal death signals that are generated on account of disturbed cellular homeostasis are inhibited through the phosphorylation of Bad. The inhibition of p53 further enhances the anti-apoptotic effects of Nef in the infected cell (Fig. 2).

**Nef genes from Indian isolates of HIV-1**

While most of the functions of Nef are conserved across the different HIV-1 subtypes, studies on Nef functions have largely been carried out with the protein from subtype B isolates, mainly the NL4-3 isolate. We have earlier reported the cloning, sequencing and phylogenetic analysis of nef genes from primary HIV-1 subtype C isolates from India. Our results also showed that in addition to env and gag, nef sequences could also be used to reliably subtype isolates belonging to the M group of HIV-1. These results have recently been confirmed by characterizing more nef sequences from Indian seropositive cases.

Besides full-length nef genes, we also cloned truncated forms with major in-frame deletions from the primary Indian isolates. Recently, we have studied the functional aspects of three of these nef alleles, F2 expressing the full-length protein, and D1 and C2 expressing proteins with in-frame deletions of critical motifs (Fig. 3A). While the F2-Nef protein was competent in downregulating cell surface CD4, MHC I and MHC II, the D1-Nef protein...

**Fig. 2.** Nef and apoptosis. Nef stimulates the expression of Fas Ligand (FasL) on the surface of HIV-infected cells through an as yet poorly characterized pathway (1). This leads to increased killing of bystander cells expressing the Fas receptor (Fas). When HIV-specific CTLs come into contact with their targets, the latter are killed through the Fas-FasL pathway. In infected cells Nef blocks this, and the TNF receptor-mediated death pathway, by inhibiting the apoptosis-associated kinase 1 (Ask1) (2). Nef directly binds p53 and prevents the induction of p53-mediated apoptosis in HIV-infected cells (3). It also unleashes the anti-apoptotic effects of Bcl-2 and Bcl-XL by PAK-mediated phosphorylation of the pro-apoptotic molecule BAD (4) causing its release from a Bcl-2 or Bcl-XL complex (5). The free Bcl-2 and Bcl-XL block cytochrome c release from the mitochondria (6). This pathway is similar to that initiated by Akt-mediated phosphorylation of BAD following cytokine binding to its cognate receptor. Full and dotted lines indicate the direct and indirect effects of Nef, respectively. (concept of figure adapted from Fig.6 of B.M. Peterlin and D. Trono. *Nat Rev Immunol* 2003; 3: 97-107).
downregulated MHC II, but not CD4 or MHC I (S.R. Das; unpublished results). The C2-Nef protein has a major deletion that encompasses the PACS-1, SH3 and PAK1/2 binding sites, and was unable to downregulate any of these surface proteins (S.R. Das; unpublished results). Surprisingly, the WL motif required for binding to CD4 was preserved in C2-Nef but it was still insufficient to downregulate cell surface CD4, suggesting that flanking sequences might also be critical. The C2-Nef protein is 121 amino acids long and is missing a large part of the core region. It is possible that this allele is non-functional and is compensated by other functional alleles in the same patient. However, its selection in a rapidly evolving viral quasi-species population is intriguing.

Structure-function relationship of the Nef protein

The variety of functions carried out by Nef is based on its ability to interact with multiple cellular proteins. These proteins and their interacting sequence motifs in Nef are shown in the Table. The HIV-2 and SIV Nef proteins are 10-30 amino acids longer compared to that of HIV-1 \(^{104}\). While it has not been possible to crystallize the full-length Nef protein, structural information has been put together based on the X-ray structure of its core domain and solution nuclear magnetic resonance (NMR) structures of its N- and C-terminal domains. The emerging picture shows a flexible N-terminal of about 70 residues followed by a well-conserved and folded core domain of about 120 amino acids. The highly conserved MGGxxS sequence at the N-terminus of Nef is myristoylated at the G2 position; this modification is critical for its attachment to the inner face of the plasma membrane and is required for all the functions associated with Nef. The G2A mutation has been shown to abrogate the CD4, MHC I and MHC II downmodulation properties of Nef. A G2A mutation in the F2-Nef protein from Indian subtype C HIV-1 also showed similar effects (S.R. Das; unpublished results).

The solution structure of Nef either alone or bound to a peptide from the cytoplasmic tail of CD4 has been determined by NMR\(^{51}\). The NMR structure of a peptide shows that the N-terminal domain of Nef forms a well-ordered alpha helix from residues 6 to 22. The core domain is the only part of the Nef protein, which has a stable tertiary structure. This fragment has been resolved by NMR\(^{51}\) and crystallography\(^{128, 129}\). It forms an \(\alpha-\beta\) domain in which a central anti-parallel \(\beta\) sheet of four strands (\(\beta\)A-\(\beta\)D) is flanked N-terminally by two long anti-parallel \(\alpha\) helices (\(\alpha\)A and \(\alpha\)B) and C-terminally by two short \(\alpha\) helices (\(\alpha\)C and \(\alpha\)D). A proline-rich sequence P69 to P78 forms a type II polyproline helix (aa 70-77), which represents the main binding site for Src family kinases through their SH3 domains. This domain is followed by two \(\alpha\) helices (aa 81-120), a four-stranded anti-parallel \(\beta\)-sheet (aa 121-186), and two additional \(\alpha\) helices (aa 187-203) \(^{104}\). Residues 60-71 and 149-180 form flexible solvent exposed loops. The three proximal helices of the Nef core domain (aa 70-120) could theoretically form a cavity accessible to drugs, which could disrupt interactions between Nef and Src family of kinases.

The motifs critical for Nef function and its binding partner are summarized in the Table. Most of the motifs that are critical for binding to different cellular proteins are in the core domain and are exposed for easy accessibility to their binding partners. A space-fill model of the core region of Nef (Fig. 3B) shows a large binding surface on one side of the protein that is responsible for MHC I downmodulation through the binding of PACS-1 and SH3 domain-containing proteins. The thioesterase-binding site or the dimerization interface is exposed as seen in view 2 (Fig. 3B); the homodimerization of Nef is stabilized by a salt-bridge between residues D123 (within the FPD motif) and R105 that forms the PAK1/2 binding site (view 1; Fig. 3B). As these residues appear to be present on different surfaces of the Nef protein, the homodimer is likely to be asymmetric. However, dimerization per se is likely to increase the collection of cellular proteins in a Nef-mediated complex. In the absence of a clear biochemical function, for example an enzymatic activity, associated with it, Nef acts as an adaptor protein and may provide a surface for bringing together critical signaling molecules within the infected cell.

Conclusion

A state of persistence favours the life cycle of HIV. Being a retrovirus, its genome is integrated into the host genome; therefore, it makes sense for HIV
### Table. Cellular proteins interacting with Nef

<table>
<thead>
<tr>
<th>Name</th>
<th>Region of Nef critical for binding/function</th>
<th>Region of the cellular partner critical for binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein modification:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-myristoyl transferase</td>
<td>MGxxxS₁</td>
<td>Unidentified 71, 72</td>
</tr>
<tr>
<td>HIV-1 protease</td>
<td>CAW↓LEA₅₅</td>
<td>Unidentified 71, 104</td>
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<tr>
<td><strong>Signalling:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Src family tyrosine kinases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hck</td>
<td>PxxPxR</td>
<td>SH3 domain 42, 107, 109</td>
</tr>
<tr>
<td>Lck</td>
<td>PxxPxR, N-terminal</td>
<td>SH3 domain 104, 128, 129</td>
</tr>
<tr>
<td>Fyn</td>
<td>PxxPxR</td>
<td>SH3 domain 104, 128, 129</td>
</tr>
<tr>
<td>Lyn</td>
<td>PxxPxR</td>
<td>SH3 domain 104, 128, 129</td>
</tr>
<tr>
<td>Vav</td>
<td>PxxPxR</td>
<td>SH3 domain 104, 128, 129</td>
</tr>
<tr>
<td>Src</td>
<td>PxxPxR</td>
<td>SH3 domain 104, 128, 129</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAK1/2 (NAK)</td>
<td>PxxPxR, MGxxxS, RR₁₀₅*, Leu₁₁₂*, FPD₁₂₁</td>
<td>Unidentified 111, 113, 124</td>
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<tr>
<td>MAPK (Erk1)</td>
<td>PxxPxR</td>
<td>SH3 domain 104, 128, 129</td>
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<tr>
<td>c-Raf1 kinase</td>
<td>DDPxxE₁₇₄</td>
<td>Unidentified 114</td>
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<tr>
<td>PI3K</td>
<td>N- and C-terminal of Nef</td>
<td>p85 subunit 42, 124</td>
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<tr>
<td>TcRζ</td>
<td>PxxPxR</td>
<td>Unidentified 42, 125</td>
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<tr>
<td>p53</td>
<td>N-terminal₁⁻₅₇</td>
<td>Unidentified 121</td>
</tr>
<tr>
<td>Ask1</td>
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<td>Unidentified 121</td>
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<td><strong>Cell surface receptors:</strong></td>
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<td></td>
</tr>
<tr>
<td>CD4</td>
<td>MGxxxS₁, WL₄₃₅, L₁₁₀*, FPD₁₂₁</td>
<td>Cytoplasmic tail 39-71</td>
</tr>
<tr>
<td></td>
<td>D/ExxxLL₁₆₅*, EE₁₅₄, DD₁₇₄</td>
<td></td>
</tr>
<tr>
<td>CD28</td>
<td>MGxxxS₁</td>
<td>Unidentified 73-75</td>
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<tr>
<td>MHCI</td>
<td>MGxxxS₁, M₂₀, EEEE₆₂, PxxPxR, FPD₁₂₁*, EE₁₅₄</td>
<td>Cytoplasmic tail 71-76-93</td>
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<tr>
<td>MHCII</td>
<td>MGxxxS₁, D/ExxxLL₁₆₅*, EEEE₆₂*, PxxPxR</td>
<td>Unidentified 894-100</td>
</tr>
<tr>
<td>** Trafficking:**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PACS-1</td>
<td>EEEE₆₂</td>
<td>Unidentified 86-88, 71</td>
</tr>
<tr>
<td>Human thioesterase (p35)</td>
<td>FPD₁₂₁</td>
<td>Unidentified 70-71</td>
</tr>
<tr>
<td>β-COP</td>
<td>EE₁₅₄</td>
<td>Unidentified 65, 66, 68, 71</td>
</tr>
<tr>
<td>Adaptor proteins AP-1/2/3</td>
<td>D/ExxxLL</td>
<td>μ subunit of AP1/2/3 55, 57-59, 63, 71</td>
</tr>
<tr>
<td>β₁ of AP-1</td>
<td>D/ExxxLL</td>
<td>β₁ of AP1 58, 59, 71</td>
</tr>
<tr>
<td>V₁H</td>
<td>DD₁₇₄</td>
<td>Unidentified 60-62, 71</td>
</tr>
</tbody>
</table>
Fig. 3. Primary sequence and structure of the HIV-1 Nef protein. (A) The amino acid sequence of a prototype Nef protein from the HIV-1 subtype B NL4-3 isolate (Nef NL) is shown and aligned to a subtype C Nef consensus sequence (Cons C), and sequences of full-length (Nef F2) and mutant (Nef D1, Nef C2) Nef proteins from Indian HIV-1 subtype C isolates. Distinct functional regions of Nef are highlighted in different colors. (B) A space-fill model of the core region of Nef is shown with distinct functional regions highlighted in different colors. View 1 shows a frontal representation of a majority of functional domains. View 2 shows a side representation highlighting the dimerization motif. View 3 is a top-down view highlighting the surface required for MHCI downmodulation through PACS-1 and SH3 domain interactions.
to promote the survival of infected cells. However, continuous expression of viral proteins at high levels also poses a risk for the infected cell to be recognized and eliminated by virus-specific CTLs. In addition, antibodies generated against HIV proteins, especially the surface proteins, would target extracellular virions, further limiting the spread of infection. The HIV counter-attack to the host immune response is to downregulate those host molecules that are critical for the development of cellular and humoral immune responses.

In this strategy, the Nef protein plays a central role by downmodulating the surface expression of CD4, MHC I, MHC II and CD28 proteins critical for the formation of an immune synapse. Lower surface levels of CD28 in infected T cells ensure that these cells cannot provide effective help following TCR engagement. Correspondingly, in professional antigen-presenting cells such as macrophages and dendritic cells that are the first cells to be infected by HIV, downmodulation of surface MHC I and MHC II ensures poor presentation of HIV peptides to TCRs on helper or cytotoxic T cells. In addition, Nef-mediated reduction in MHC I expression on the surface of HIV-infected cells helps these cells escape from virus-specific CTLs. The overall effect of this strategy is to reduce T cell help for generating effective virus-specific antibody and cytotoxic responses, as well as reduce the engagement of infected cells and virus-specific CTLs. The Nef protein also promotes the survival of infected cells by inhibiting "outside-in" as well as "inside-in" death signals; in the latter case, p53-independent as well as dependent pathways are invoked.

Besides an active role in promoting viral persistence, Nef initiates a transcriptional programme in T cells similar to that seen in TCR-activated T cells. This has an overall effect of enhancing viral replication and virion infectivity. The Nef protein interacts with a wide range of cellular proteins, many known to perform critical functions in signaling pathways. Structure-function studies on the Nef protein have identified a number of motifs involved in protein-protein interactions and favour a model wherein Nef acts as an adaptor to accumulate signaling complexes in the infected cell. The downstream effects of these signaling pathways position Nef as a critical “positive factor” in HIV pathogenesis.

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