AKI MANNINEN

Cellular Functions of the Human Immunodeficiency Virus Type 1 (HIV -1) Nef Protein

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1. LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by the roman numerals:

I Manninen A., Hiipakka M., Vihinen M., Lu W., Mayer B.J, Saksela K. **SH3-domain binding function of HIV-1 Nef is required for association with a PAK-related kinase.** *Virology* **250**: 273-282, 1998

II Renkema G-H., Manninen A., Mann D.A., Harris M., Saksela K. **Identification of the Nef-associated kinase as p21-activated kinase 2.** *Curr Biol* **9**: 1407-1410, 1999

III Renkema G-H., Manninen A., Saksela K. **Human immunodeficiency virus type-1 Nef selectively associates with a catalytically active subpopulation of p21-activated kinase 2 (PAK2) independently of PAK2 binding to Nck or β-PIX.** *J Virol* **75** (5): 2154-2160, 2001

IV Manninen A., Renkema G-H., Saksela K. **Synergistic activation of NFAT by HIV-1 Nef and the Ras/MAPK pathway.** *J Biol Chem* **275** (22): 16513-16517, 2000

V Manninen A., Huotari P., Hiipakka M,.Renkema G-H., Saksela K. **Activation of NFAT-dependent gene expression by Nef: conservation among divergent Nef alleles, dependence on SH3-binding and membrane association, and cooperation with protein kinase C-θ.** *J Virol* **75** (6): 3034-3037, 2001

VI Manninen A., Saksela K. **HIV-1 Nef interacts with IP_3-receptor to activate calcium signaling.** Submitted 2001

2. ABBREVIATIONS

β-COP	β-subunit of COP I coatomer
2-APB	2-aminoethoxydiphenyl borate
AIDS	Acquired immunodeficiency syndrome
AP-1	Activator protein-1
AP-2	Adaptor protein complex 2
ARRE-2	Antigen receptor response element of the interleukin-2 gene
ATP	Adenosine 5'-trisphosphate
BSA	Bovine serum albumin
CA	Capsid protein
CCE	Capacitative calcium entry
cDNA	Double-stranded DNA copy of the viral RNA
CMV	Cytomegalovirus
CN	Calcineurin
CRIB-motif	Cdc42/Rac-interactive binding motif
CsA	Cyclosporin A
CTL	Cytotoxic T lymphocyte
DAG	Diacylglycerol
DN	Dominant-negative
DTT	Dithiotreitol
EDTA	Ethylenediamine N,N,N',N' tetraacetic acid
EGTA	Ethyleneglycol-bis-β-aminoethyl ether N,N,N',N' tetra-acetic acid
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GEF	Guanosine nucleotide exchange factor
gp	Glycoprotein
GST	Glutathione-S-transferase
GTP	Guanosine 5'-trisphosphate
HA	Hemagglutinin
HAART	Highly active anti-retroviral therapy
HEPES	N-2-hydroxyethylpiperazine-N´-2 ethanosulfonic acid
HIV	Human immunodeficiency virus
HSV	Herpes virus saimiri
hTEII	Human thioesterase II
HTLV	Human T cell leukemia virus
IL	Interleukin
IN	Integrase
IP_3R	Inositol trisphosphate receptor
ITAM	Immunoreceptor tyrosine-based activation motif
IVKA	*In vitro* kinase assay
JNK/p38	Jun N-terminal kinase/p38 mitogen-activated protein kinase
kb	Kilobase
kDa	KiloDalton
LAT	Linker for activation of T cell
LTR	Long terminal repeat
MA	Matrix protein
MAPK	Mitogen-activated protein kinase
MBP	Maltose-binding protein
MHC I	Major histocompatibility complex class I
MIP	Macrophage inflammatory protein
mRNA	Messenger-ribonucleic acid
NAK	Nef-associated kinase
NC	Nucleocapsid protein
Nef	Negative factor
NF-κB	Nuclear factor κB
NFAT	Nuclear factor of activated T cells
NMR	Nuclear magnetic resonance
ONPG	O-nitrophenyl-β-D-galactopyranoside
PAK	p21-activated kinase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PI3-K	Phosphatidylinositol 3-kinase
PIC	Pre-integration complex
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIX/Cool	Pak-interactive exchange factor/ cloned-out of library
PKC	Protein kinase C
PLC	Phospholipase
PMA	Phorbol 12-myristate, 13-acetate
PMSF	Phenylmethylsulfonylfluoride
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo-[3,4-d]-pyrimidine
PPII-helix	Polyproline type 2 helix
PR	Protease
Rev	Regulator of viral protein expression
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecylsulphate polyacryl-amide gel electrophoresis
SH	Src-homology
SIV	Simian immunodeficiency virus
SLP-76	SH2 domain-containing leucocyte protein of 65 kDa
SMAC	Supramolecular activation cluster
SOC	Store-operated calcium channel
SPR	Surface plasmon resonence
SRE	Serum response element
TAT	Transactivator protein
TCR	T cell receptor
TNF	Tumour necrosis factor
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WM	Wortmannin
ZAP-70	Zeta-associated protein-70

3. INTRODUCTION

Nef is a 25-34 kilodalton (kDa) accessory protein of the primate lentiviruses (HIV-1/2 and SIV). Despite being dispensable for viral life cycle in most transformed cell lines, Nef has an essential role *in vivo* by promoting efficient viral replication and subsequent evasion of host immune defence to develop AIDS. Several cellular functions of Nef, downregulation of the surface expression of CD4 and major histocompatibility complex class I (MHC I) molecules, enhancement of viral replication kinetics as well as intrinsic infectivity of HIV particles, have been described. In addition, Nef has been shown to modulate cellular signal transduction pathways, an effect that may contribute to the functions of Nef described above.

The molecular mechanisms of Nef-mediated downregulation of CD4 and MHC I molecules are relatively well understood, but correlating these functions with the pathogenesis of AIDS has not been straightforward. On the other hand, although enhanced HIV virion infectivity and kinetics of virus replication could readily explain the pathogenesis-promoting effects of Nef, the molecular mechanisms of these functions have remained elusive. A number of interesting connections between Nef and various cellular signaling proteins have been reported but the direct relevance of these interactions for the positive effects of Nef on viral replication has not been proven. Thus, although Nef seems to exert its effects by interacting with host proteins, the mechanisms have not yet been fully elucidated at a molecular level.

The purpose of this work was to set up model systems and use them to study the effects of Nef on cellular signaling pathways, especially in T cells. Another goal was to characterize Nef-interacting cellular proteins and the molecular mechanisms of such interactions in order to assess their relevance for Nef functions.

4. REVIEW OF LITERATURE

4.1. Human immunodeficiency virus

Introduction

In the early 1980's Luc Montagnier and Robert Gallo independently identified a novel retrovirus as a possible cause of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al.* 1983, Gallo *et al.* 1983). The two groups called their viruses lymphadenopathy-associated virus (LAV) and human T cell leukaemia virus III (HTLV-III) respectively. These findings were followed by characterization of an AIDS-associated retrovirus (ARV) by Levy and co-workers (Levy *et al.* 1984). Although sharing some characteristics with human T cell leukaemia viruses (HTLV), these novel retroviruses were shortly shown to have several properties quite distinct from HTLV and were all recognized as members of the *Lentivirinae* family. Subsequently, they were collectively named human immunodeficiency viruses (HIV) (Coffin *et al.* 1986). HIV-infected individuals develop severe defects in their immune systems and suffer from opportunistic infections and various forms of cancer. The eventually fatal immune dysfunction is due to a gradual depletion of CD4-positive T helper cells, an indicative of a progressive disease, that leads to perturbed functions of cytotoxic T cells (CTLs) and B lymphocytes. Now after twenty years of HIV research, despite remarkable advances in understanding the molecular biology of HIV and development of antiviral drugs, more than 30 million people, (according to World Health Organization), live with HIV/AIDS worldwide. A highly active anti-retroviral therapy (HAART) has been successively used to restrict virus replication in HIV-infected patients. However, current combinations of drugs presumably cannot eradicate the virus and thus require a life-long treatment that poses a risk for development of drug-resistant HIV mutants. Moreover, these drug regimens are expensive and not well tolerated by all patients, which indicates that novel approaches in drug development are required (for a review of HAART see Bonfanti *et al.* 1999, Shafer and Vuitton 1999, Crowe and Sonza 2000).

Molecular characteristics of HIV-1

Human immunodeficiency virus type 1 (HIV-1), along with related HIV-2 and simian immunodeficiency viruses (SIV) belong to primate lentiviruses (*Lentivirinae*) that are a subgroup of the retrovirus (*Retroviridae*) family. The genome of HIV-1 consists of a 9.7 kb long single-stranded RNA molecule (Figure 1). The organization of the HIV-1 genome is complex as all three reading frames are used in a partially overlapping manner to encode for nine genes. These genes can be divided into two groups: structural genes common to all retroviruses (Figure 1, light bars) and regulatory genes (dark bars) that aid viral replication at different stages of viral life cycle.

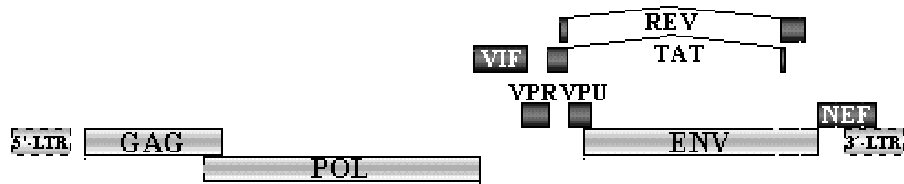

Figure 1. Schematic representation of the HIV-1 genome

The primary transcript of HIV-1 is a full-length viral mRNA that is translated into Gag- and Pol-precursor proteins. These precursor proteins are proteolytically cleaved: Gag into capsid (CA or p24), matrix (MA), p6 and nucleocapsid (NC) proteins and Pol into virus-specific enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN). The precursor protein gp160 is encoded by a singly spliced message from the full-length viral mRNA. gp160 is processed into envelope (Env) proteins gp120 and gp41. The regulatory proteins are translated from different multiply spliced mRNAs (Levy 1998).

Virion structure of HIV-1

The virion structure of HIV-1 is schematically shown in figure 2 (for a review see Turner and Summers 1999). The outermost surface consists of a lipid bilayer to which the envelope proteins or spikes of the virus are embedded. The lipid bilayer originating from virus producing host cell also contains several cellular membrane proteins (Arthur *et al.* 1992). A matrix shell, formed by multimerized matrix proteins, lines the inner surface of the viral membrane. Inside the matrix layer is the conical capsid core which encapsidates two copies of the unspliced viral RNA genome. Oligomerized nucleocapsid proteins stabilize the genome. Virus-specific enzymes: RT, IN and PR are associated with the ribonucleoprotein complex. Of the regulatory proteins, viral protein R (Vpr) is likely to be closely associated with the matrix layer (Lu *et al.* 1993). Viral infectivity factor (Vif) (Liu *et al.* 1995, Camaur and Trono 1996) and Negative factor (Nef) (Welker *et al.* 1996) are also associated with viral particles whereas the three remaining regulatory proteins, regulator of viral protein expression (Rev), transactivator protein (Tat) and viral protein U (Vpu) do not appear to be packaged into virions.

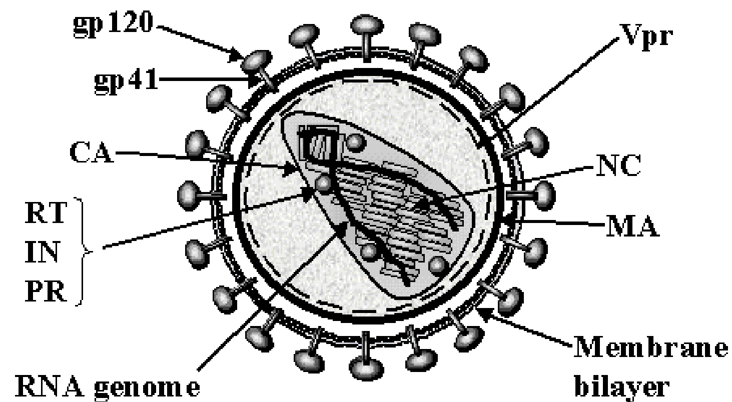

Figure 2. Morphology of HIV virion

Functions of HIV-1 proteins

The roles of the structural proteins in constructing the basic architecture of virions and providing the key enzymatic functions required for viral life cycle are more readily understood, whereas the roles of the six regulatory proteins in HIV infection have remained partially unresolved. Two of them, namely Tat (reviewed in Karn 1999) and Rev (reviewed in Hope 1999), are prerequisites for viral life cycle. Tat stimulates transcription from proviral long terminal repeat by facilitating the elongation of viral mRNAs. The Rev protein regulates the transport of unspliced mRNAs from the nucleus to the cytoplasm and thus enables the translation of structural proteins from full-length viral mRNAs. This event allows the production of new progeny viruses and determines the shift from early to late phase in the viral replication cycle.

The remaining four regulatory proteins, Vif, Vpr, Vpu and Nef are not essential for viral replication *in vitro*, but they have been shown to be required for efficient viral replication and full pathogenicity *in vivo*. Because of their dispensability *in vitro*, these factors have been called accessory proteins. The Vif protein has been implicated in the stabilization of newly synthesized virion DNA intermediates during the late stages of viral assembly (Simon and Malim 1996). Additionally, Vif may have a role in the nuclear transport of the preintegration complex (PIC) (Karczewski and Strebel 1996). The primary functions of Vpr are targeting of the PIC to the nucleus in non-dividing cells (Heinzinger *et al.* 1994, Yao *et al.* 1995) and induction of an arrest in the G_2 phase of the cell cycle (Di Marzio *et al.* 1995, He *et al.* 1995, Jowett *et al.* 1995, Re *et al.* 1995). Vpu induces degradation of the

CD4 molecule in the endoplasmic reticulum (Willey *et al.* 1992, Bour *et al.* 1995) and enhances release of virions from infected cells, presumably by forming ion channels in cell membranes (Gottlinger *et al.* 1993, Ewart *et al.* 1996, Schubert *et al.* 1996). The Nef protein with its pleiotropic effects is the subject of this study and will be discussed in detail in the chapter below.

Replication cycle of HIV-1

A schematic representation of the HIV life cycle is shown in figure 3. The viral envelope glycoprotein gp120 specifically binds to a CD4 molecule on the surface of a target cell (Capon and Ward 1991). For efficient entry and infection lentiviruses also require additional cell surface molecules, and recently chemokine receptors were identified as functional co-receptors for HIV (reviewed in Berger *et al.* 1999). These interactions trigger a series of conformational changes in gp120, which are likely to result in exposure of the fusion peptide in the gp41 protein and subsequent fusion of viral and cellular membranes (Berger *et al.* 1999). Following the fusion of membranes, the nucleocapsid enters the cytoplasm. The viral RNA, still enclosed in the viral capsid, is uncoated in a process aided by capsid proteins (p24) and cellular proteins called cyclophilins (Luban *et al.* 1993). Uncoating is followed by reverse transcription of the viral RNA, generating a double-stranded DNA copy (cDNA) of the genome (reviewed in Gotte *et al.* 1999). The resulting preintegration complex consisting of at least cDNA, MA, Vpr and IN proteins is transported into the nucleus, where integration of the viral genome into host chromosome takes place (Esposito and Craigie 1999, Hindmarsh and Leis 1999). Following virus integration, the transcription of HIV genes begins and in the early phase Tat, Rev and especially Nef mRNA expression predominate (Kim *et al.* 1989, Robert-Guroff *et al.* 1990, Greene 1991). Eventually, accumulating levels of Rev protein result in a shift from the expression of the regulatory proteins to the structural proteins. The assembly of the new virus progeny takes place at the plasma membrane, where viral RNA genome is packaged into capsids that bud off from the membrane along with the viral envelope proteins (Garnier *et al.* 1998). Cellular cyclophilins may serve a crucial function also during the assembly (Luban 1996). The HIV life cycle's final event is the maturation of cell-free particles upon action of virion-incorporated PR (Navia and McKeever 1990). These matured viruses, if assembled correctly, are then capable of productive infection when they encounter appropriate target cells.

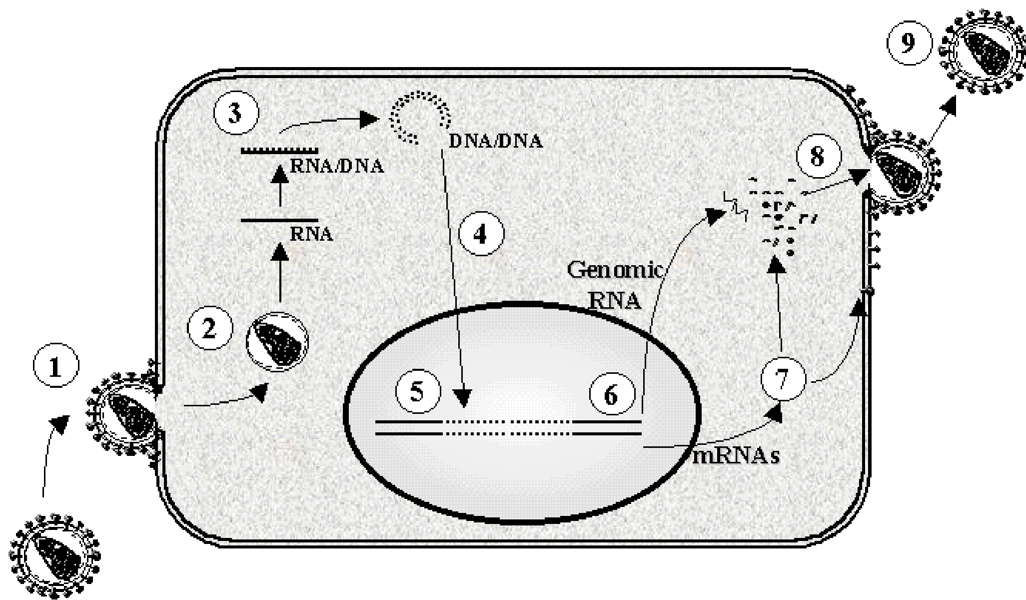

Figure 3. Viral life cycle of HIV

1. Attachment and entry **2.** Uncoating **3.** Reverse transcription (— RNA, - - -DNA) **4.** Nuclear transport of the preintegration complex **5.** Integration **6.** Transcription **7.** Translation and transport of viral proteins to the plasma membrane **8.** Assembly and budding **9.** Maturation. Adapted from (Levy 1998).

HIV-2 and SIV

Shortly after the discovery of HIV-1, Clavel *et al.* found another AIDS-associated virus in Africa, which turned out to be surprisingly different from HIV-1 (Clavel *et al.* 1986, Guyader *et al.* 1987). This new type of HIV virus, named HIV-2, causes a similar disease but seems to be less pathogenic. Moreover, some investigators believe that HIV-2 is also less contagious, which may explain why it has not spread as extensively as HIV-1 (Li *et al.* 1998a, Mansinho 1999).

Simian immunodeficiency viruses (SIV) are a large and complex group of lentiviruses (Hirsch *et al.* 1995). Some SIV isolates are capable of inducing an AIDS-like disease in certain primate species, other than their original host. Such models have been used to study the *in vivo* pathogenesis of immunodeficiency viruses (reviewed in Geretti 1999).

4.2. The Nef protein

Nef is a small (27-34 kDa) myristoylated protein unique to primate lentiviruses that, despite being dispensable for viral replication *in vitro*, has been shown to have a critical role in the pathogenesis of AIDS. The first piece of evidence for this came from studies on simian immunodeficiency virus (SIV) when Kestler *et al.* demonstrated that macaques inoculated with SIV viruses containing a deleted Nef gene neither developed high viremia nor simian AIDS (Kestler *et al.* 1991). Subsequently, several groups have reported similar phenomenon in human patients infected with Nef-deficient HIV-1 viruses. These individuals have significantly delayed onset of the symptoms or have remained asymptomatic for up to 20 years (Deacon *et al.* 1995, Kirchhoff *et al.* 1995, Salvi *et al.* 1998, Learmont *et al.* 1999). Interestingly, Hanna *et al.* recently showed that in a transgenic mouse model, expression of Nef alone was sufficient to induce an AIDS-like disease (Hanna *et al.* 1998). Although the crucial role of Nef for viral replication *in vivo* has been recognized for years, the underlying molecular mechanisms remain unclear. Addressing this question is complicated due to the fact that Nef-defective viruses replicate efficiently in most transformed cell lines. However, *in vitro* studies have revealed several cellular functions for Nef especially highlighting its capacity to modulate cellular signaling pathways.

4.2.1 Cellular functions of Nef

Downregulation of CD4 and Major Histocompatibility Complex class I (MHC I) molecules

In 1987 Guy and co-workers observed that expression of HIV-1 Nef downregulated the surface expression of CD4 (Guy *et al.* 1987). This event was subsequently confirmed by others and was found to be a consequence of lysosomal transport and degradation of CD4 (Garcia and Miller 1991, Anderson *et al.* 1993, Garcia *et al.* 1993, Mariani and Skowronski 1993). The molecular mechanism of this function is now relatively well understood. It seems that Nef regulates the surface expression of CD4 at multiple levels. The cytoplasmic tail of the CD4-molecule contains a dileucine motif that has been implicated as a universal sorting signal for endocytotic transport (reviewed in Kirchhausen *et al.* 1997, Le Borgne and Hoflack 1998). In T cells, CD4 is maintained at the cell surface by Src-family tyrosine kinase Lck that, by binding to the dileucine motif in CD4, prevents its recognition by the endocytotic machinery (reviewed in Marsh and Pelchen-Matthews 1996). Protein kinase C (PKC)-mediated phosphorylation of serines, proximal to the dileucine motif, induces both dissociation of Lck and interactions with the AP-2 clathrin adaptor complex. The CD4-molecule is subsequently rapidly internalized and degraded in the lysosomes (Marsh and Pelchen-Matthews 1996). Nef has been shown to interact with the cytoplasmic tail of CD4-molecules in a dileucine motif-dependent manner (Aiken

et al. 1994, Bandres *et al.* 1995, Salghetti *et al.* 1995, Gratton *et al.* 1996, Grzesiek *et al.* 1996a, Rossi *et al.* 1996, Preusser *et al.* 2001). Therefore, by binding to the dileucine motif, Nef disrupts Lck/CD4-interaction (Salghetti *et al.* 1995). Consequently, the internalization signal in CD4 is exposed (Garcia and Miller 1991, Aiken *et al.* 1994, Bandres *et al.* 1995, Salghetti *et al.* 1995).

In the absence of serine phosphorylation, however, internalized CD4 is recycled from the early endosomes back to the plasma membrane (Marsh and Pelchen-Matthews 1996). Interestingly, Mangasarian and co-workers demonstrated that substituting Nef for the cytoplasmic domains of CD4 or CD8 targeted the resulting chimeras for rapid endocytosis and lysosomal degradation (Mangasarian *et al.* 1997). As these chimeras do not contain the endogenous internalization signals of CD4 or CD8, this data indicated that Nef has an internalization signal of its own. In other words, Nef can target CD4 for endocytosis and subsequent degradation by serving as a link between CD4 and components of the endocytotic machinery. It was subsequently shown that Nef interacts with the medium (μ) subunits of clathrin adaptor complexes (Greenberg *et al.* 1997, Le Gall *et al.* 1998, Piguet *et al.* 1998). Unexpectedly, Nef proteins from HIV-1 and HIV-2/SIV appear to have evolved different strategies in binding to these adaptor complexes. A highly conserved dileucine-based motif in the C-terminal disordered loop of HIV-1 Nef is critical for binding to adaptors (Bresnahan *et al.* 1998, Craig *et al.* 1998, Greenberg *et al.* 1998a). In contrast, the corresponding motif seems to have a more minor role for HIV-2/SIV Nefs (Piguet *et al.* 1998, Bresnahan *et al.* 1999). An N-terminal sequence, that resembles a tyrosine-based internalization motif and is not present in HIV-1 Nef alleles, plays an additional role for HIV-2/SIV Nefs (Piguet *et al.* 1998, Bresnahan *et al.* 1999). The tyrosine-based internalization motif, like the dileucine motif, mediates endocytosis by interacting with adaptor complexes (Kirchhausen *et al.* 1997, Le Borgne and Hoflack 1998).

Certain studies have suggested that coupling of CD4 via Nef to the clathrin adaptor complexes is not sufficient for lysosomal targeting (Kim *et al.* 1999, Piguet *et al.* 1999). Nef has been reported to bind to the β-subunit of COP I coatomer (β-COP) (Benichou *et al.* 1997, Piguet *et al.* 1999, Janvier *et al.* 2001). Piquet *et al.* proposed a model where Nef sequentially interacts with the adaptor complexes to internalize CD4 into early endosomes and with β-COP to target CD4 for lysosomal degradation (Piguet *et al.* 1999).

The capacity of Nef to bind to the human thioesterase II (hTE II) has been shown to correlate with Nef-mediated CD4-downregulation (Benichou *et al.* 1997, Liu *et al.* 1997, Watanabe *et al.* 1997). However, not all Nef variants bind to hTE II with high affinity (Cohen *et al.* 2000).

Vma13p, a catalytic subunit of the universal proton pump (vacuolar proton ATPase) was found in a yeast two-hybrid screen to bind to Nef (Lu *et al.* 1998). A di-acidic motif consisting of two aspartates was found to be crucial for both the ability of Nef to downregulate CD4 and to associate with Vma13p (Lu *et al.* 1998). As Vma13p itself interacts with adaptor complexes it might provide an alternative connection between Nef/CD4 and these complexes. The vacuolar proton ATPase is responsible for acidifying intracellular organelles. Therefore, it is possible that Nef/Vma13p-interaction affects the formation and acidification of endosomes and lysosomes. To this end, Nef has been shown to increase the plasma membrane area occupied by clathrin-coated structures, as well as the number of endosomes and lysosomes (Foti *et al.* 1997, Sanfridson *et al.* 1997).

Nef-mediated downregulation of CD4 could enhance virus replication by several mechanisms. Rapid internalization of the HIV-receptor may prevent potentially harmful superinfection of productively infected cells (Benson *et al.* 1993). Moreover, reduced levels of the surface expression of CD4 can facilitate the release and/or enhance the infectivity of newly synthesized virions from the producer cells (Bour *et al.* 1999, Lama *et al.* 1999, Ross *et al.* 1999). Furthermore, the immunological defects resulting from rapid loss of cell surface CD4 in infected cells may provide indirect mechanisms by which Nef could promote viral replication *in vivo*. In addition to Nef, Env and Vpu are independently capable of down-modulation of CD4 (Chen *et al.* 1996). Therefore, this phenomenon is likely to be important for optimal virus replication (Chen *et al.* 1996).

Nef has also been shown to downregulate the surface expression of MHC I (Schwartz *et al.* 1996). Surprisingly, the molecular mechanism of Nef-induced MHC I-downregulation fundamentally differs from that of CD4-downregulation and was shown to be due to retention of MHC I in the *trans*-Golgi compartment (Greenberg *et al.* 1998a, Greenberg *et al.* 1998b, Le Gall *et al.* 1998, Mangasarian *et al.* 1999). As with the CD4-downregulation function of Nef, the molecular mechanisms underlying MHC I-trafficking by HIV-1 and SIV Nef alleles are different (Swigut *et al.* 2000). An interaction between HIV-1 Nef and cytosolic sorting protein PACS-1 has been reported to be required for this function (Piguet *et al.* 2000). By reducing the abundance of MHC I on the plasma membrane Nef reportedly protects infected cells against cytotoxic T lymphocyte (CTL)- and natural killer (NK) cell-mediated killing (Collins *et al.* 1998, Cohen *et al.* 1999).

Enhancement of HIV infectivity and the kinetics of virus replication

Although initially named as a negative factor (Nef) because of early studies that described an inhibitory role of Nef in HIV replication (Ahmad and Venkatesan 1988, Cheng-Mayer *et al.* 1989), this hypothesis was later disproved by several groups who demonstrated the positive effects of Nef on viral replication both *in vivo* and *in vitro* (Kestler *et al.* 1991, de Ronde *et al.* 1992, Zazopoulos and

Haseltine 1993, Jamieson *et al.* 1994). Nef was found to enhance the infectivity of virions and the kinetics of viral replication (Chowers *et al.* 1994, Miller *et al.* 1994, Spina *et al.* 1994). The Nef phenotype was most pronounced when a low viral input was used to infect resting peripheral blood mononuclear cell (PBMC) cultures followed by activation with mitogen (Miller *et al.* 1994, Spina *et al.* 1994). After entering the target cells, the viral genomes associated with wild-type Nef (+) virions underwent reverse transcription more efficiently than those incorporated into Nef-deficient virions (Aiken and Trono 1995, Chowers *et al.* 1995, Schwartz *et al.* 1995). It was shown that the Nef-mediated enhanced virion infectivity and the CD4-downregulation by Nef were separate functions (Goldsmith *et al.* 1995, Saksela *et al.* 1995).

Several possible mechanisms could explain the Nef-mediated induction of the synthesis of proviral DNA. Nef is incorporated into virions (Pandori *et al.* 1996, Welker *et al.* 1996, Welker *et al.* 1998, Kotov *et al.* 1999) and could thus directly assist the reverse transcription process. The virion-associated Nef is processed by viral protease into a truncated form that may have specific functions (Pandori *et al.* 1996, Welker *et al.* 1996). However, evidence opposing such a hypothesis has been reported (Miller *et al.* 1997, Chen *et al.* 1998, Pandori *et al.* 1998). In addition, the resulting truncated Nef is likely to be inactive in all known cellular functions of Nef (Aiken *et al.* 1994, Chowers *et al.* 1994, Wiskerchen and Cheng-Mayer 1996). Therefore, the biological significance of proteolytic processing of virion-associated Nef remains to be proven. Moreover, the reduced infectivity of Nef-defective virions cannot be complemented by ectopic expression of Nef in the target cells arguing against a direct role for Nef in proviral DNA synthesis (Aiken and Trono 1995, Miller *et al.* 1995, Pandori *et al.* 1996). Furthermore, while up to 80 per cent of the mRNA species synthesized from the newly integrated provirus encode for Nef (Robert-Guroff *et al.* 1990; Ranki *et al.* 1994), only 10-100 Nef molecules are incorporated into each virion (Pandori *et al.* 1996, Welker *et al.* 1996, Welker *et al.* 1998, Kotov *et al.* 1999).

In light of the above-mentioned studies, it seems likely that the critical function of Nef is exerted at an early post-integration step rather than in the context of a mature virion. Fitting to such a scenario, Swingler *et al.* reported that Nef expression enhanced incorporation of a cellular serine kinase to the virion (Swingler *et al.* 1997). This correlated with enhanced serine phosphorylation of the viral matrix protein (MA) (Swingler *et al.* 1997). Although the role of the serine phosphorylation of MA is elusive, it has been suggested that this modification is required for optimal virion infectivity (Bukrinskaya *et al.* 1996).

Nef may also enhance the viral infectivity by modulating the cellular environment in infected cells to be optimal for efficient and accurate assembly of the new progeny virions. For example, Nef-mediated changes in cellular signaling could alter post-translational modification of viral components. Furthermore, Lama and

co-workers recently reported that due to Nef-mediated downregulation of CD4, Nef (+) virions produced from cells expressing high levels of CD4 were more infectious than those produced in the absence of Nef (Lama *et al.* 1999). The Nef (-) virions failed to efficiently incorporate gp120 envelope protein (Lama *et al.* 1999). In contrast, Ross *et al.*, by using essentially the same experimental system, concluded that virions produced in the absence of Nef, resulted in lower yields but equal infectivity (Ross *et al.* 1999). However, earlier reports have demonstrated a difference in virion infectivity between Nef (+) and Nef (-) viruses produced in CD4-negative cells (Miller *et al.* 1994) and shown that CD4-downregulation and enhancement of viral infectivity are independent functions of Nef (Goldsmith *et al.* 1995, Saksela *et al.* 1995, Wiskerchen and Cheng-Mayer 1996, Hua *et al.* 1997, Iafrate *et al.* 1997). These data indicate that Nef-mediated CD4-downregulation can only partially mediate the positive effect of Nef on HIV particle infectivity.

4.2.2. Modulation of cellular signaling pathways by Nef

In addition to CD4-downregulation and enhanced HIV-infectivity functions, Nef has been shown to modulate cellular signaling pathways. Because of contradictory results and incomplete understanding of the signaling pathways implicated in Nef studies, the exact mechanisms and consequences of this function of Nef remain unresolved. Several issues could contribute to the observed variation in results: the choice of the Nef allele, the cellular study system (fibroblasts vs. lymphocytes, primary cells vs. different immortalized cell lines) and cell culture conditions (Renkema and Saksela 2000). Moreover, because Nef is capable of interacting with a plethora of cellular proteins, many of which are involved in signaling, the levels as well as the duration of Nef expression are likely to contribute to the cellular response to Nef. In this respect, it is also important to consider the effects of Nef on the expression patterns of cell surface molecules with signaling capacity, such as CD4, as downregulation of these molecules might indirectly interfere with cellular signaling.

In order to understand the molecular mechanisms by which Nef modulates signal transduction, several studies have aimed at identification of Nef-interacting cellular proteins. Some clues for such interactions can be found by studying the structure of the Nef protein, especially by comparing different Nef variants with each other. Although the Nef proteins from HIV-1 and HIV-2/SIV seem to have similar cellular functions, their amino acid sequences are considerably less well conserved (~50-60% identity). Recent data indicate that HIV-1 and HIV-2/SIV Nef proteins have evolved different molecular mechanisms for some of the common functions (Howe *et al.* 1998, Bresnahan *et al.* 1999, Cheng *et al.* 1999, Greenway *et al.* 1999, Xu *et al.* 1999, Carl *et al.* 2000, Swigut *et al.* 2000). Perhaps for this reason, some of the structural motifs conserved among HIV-1 strains may have a less significant role or be completely absent from HIV-2 or

SIV Nef and vice versa. Nevertheless, several regions in HIV-1 and HIV-2/SIV are highly conserved, suggesting an important role for these motifs in mediating the crucial functions of Nef.

The proline-rich sequence (PxxP-motif) in the core domain of Nef is one such example. The PxxP-motif is a minimal consensus sequence found in the ligands of Src homology 3 (SH3) domains (Cicchetti *et al.* 1992, Ren *et al.* 1993). The SH3/PxxP-pairs serve as mediators of protein-protein interactions and are particularly abundant among cellular signaling proteins (Pawson 1994, Lim 1996, Dalgarno *et al.* 1997, Mayer and Gupta 1998, Sudol 1998). An intact PxxP-motif in Nef is required for enhanced virus replication and particle infectivity, as well as for other Nef functions - with the notable exception of the downregulation of CD4 (Goldsmith *et al.* 1995, Saksela *et al.* 1995, Wiskerchen and Cheng-Mayer 1996, Iafrate *et al.* 1997, Khan *et al.* 1998, **I**). Interestingly, several SH3-containing proteins have been reported to bind to Nef (see below).

N-terminal-myristylation and an adjacent cluster of basic amino acids is another conserved domain of Nef. This bipartite motif that is reminiscent of the so-called SH4 domain (Resh 1994) mediates association of Nef with cellular membranes (Kaminchik *et al.* 1991). Approximately 50% of Nef expressed in cells is membrane-bound (Kaminchik *et al.* 1994, Welker *et al.* 1996). Targeting of Nef to the plasma membrane has been shown to be required for virtually all of its functions (Aiken *et al.* 1994, Chowers *et al.* 1994, Wiskerchen and Cheng-Mayer 1996). However, certain data suggest that the cytosolic Nef may have special functions different from those of the membrane-associated Nef (Baur *et al.* 1994). In some circumstances, Nef seems to localize in the nucleus but the relevance of these findings remains unknown (Murti *et al.* 1993, Ranki *et al.* 1994).

A striking example of the capacity of Nef to modulate cellular signaling is provided by observations of Nef-mediated malignant transformation of immortalized murine fibroblasts (Du *et al.* 1995, Briggs *et al.* 1997). Expression of the SIVpbj14 Nef variant in murine 3T3 fibroblasts led to morphological transformation of these cells (Du *et al.* 1995). pbj14 is a rare SIV strain, which causes an acute disease and death in infected macaques (Fultz *et al.* 1989). This phenotype of pbj14 Nef seems to depend solely on an immunoreceptor tyrosine-based activation motif (ITAM) located close to the aminoterminus (Luo and Peterlin 1997). However, ITAM-based activation seems to be a special feature of pbj14 Nef because such motifs have not been found in other Nef variants. Briggs *et al.* reported that co-expression of a native HIV-1 Nef together with a cellular proto-oncogene Hck transformed Rat2 fibroblasts (Briggs *et al.* 1997). This was due to Nef-mediated activation of Hck (Briggs *et al.* 1997). In addition, Nef has been shown to affect signaling in other non-lymphoid cell systems (De and Marsh 1994, Graziani *et al.* 1996, Romero *et al.* 1998, Fackler *et al.* 1999, Kohleisen *et al.* 1999, Plemenitas *et al.* 1999). The natural hosts supporting the majority of

HIV replication *in vivo* are T lymphocytes where the cellular functions of Nef were initially described. Therefore, the effects of Nef on T cell signaling are likely to be relevant for the pathogenesis-promoting functions of Nef.

T cell receptor (TCR) signaling

Signals emanating from T cell receptor (TCR)-complex are implicated in regulation of all of the major aspects of T lymphocyte function (Figure 4). Physiological stimulation of T cells by antigens via TCR is followed by a clustering of TCRs and formation of supramolecular activation clusters (SMACs) (Penninger and Crabtree 1999). Src-family tyrosine kinases, Lck and Fyn, are recruited to these specialized plasma membrane domains (Penninger and Crabtree 1999). Subsequent activation of Lck and Fyn leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the TCR-components (Clements *et al.* 1999). Phosphorylated ITAMs recruit zeta associated protein-70 (ZAP-70) tyrosine kinases that become activated. Upon ZAP-70/Lck-mediated phosphorylation, adaptor molecules Linker for activation of T cell (LAT) and SH2 domain-containing leucocyte protein of 65 kDa (SLP-76) serve as platforms for highly orchestrated assemblies of large heterogenous protein complexes consisting of for example, other adaptor proteins, serine/threonine kinases, small GTPases and their guanine nucleotide exchange factors (GEFs) (Qian and Weiss 1997, van Leeuwen and Samelson 1999). These complexes integrate signals from the plasma membrane receptors and convey them to appropriate activation of various downstream signaling cascades, most notably the mitogen-activated protein kinase (MAPK) cascade and the Ca^{2+}/calcineurin (CN) cascade. An important intermediate in TCR-triggered activation of both of these cascades is phospholipase Cγ-1 (PLCγ1) which catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These in turn regulate Protein kinase C (PKC) activity and intracellular calcium levels, respectively (Guse 1998, Carpenter and Ji 1999).

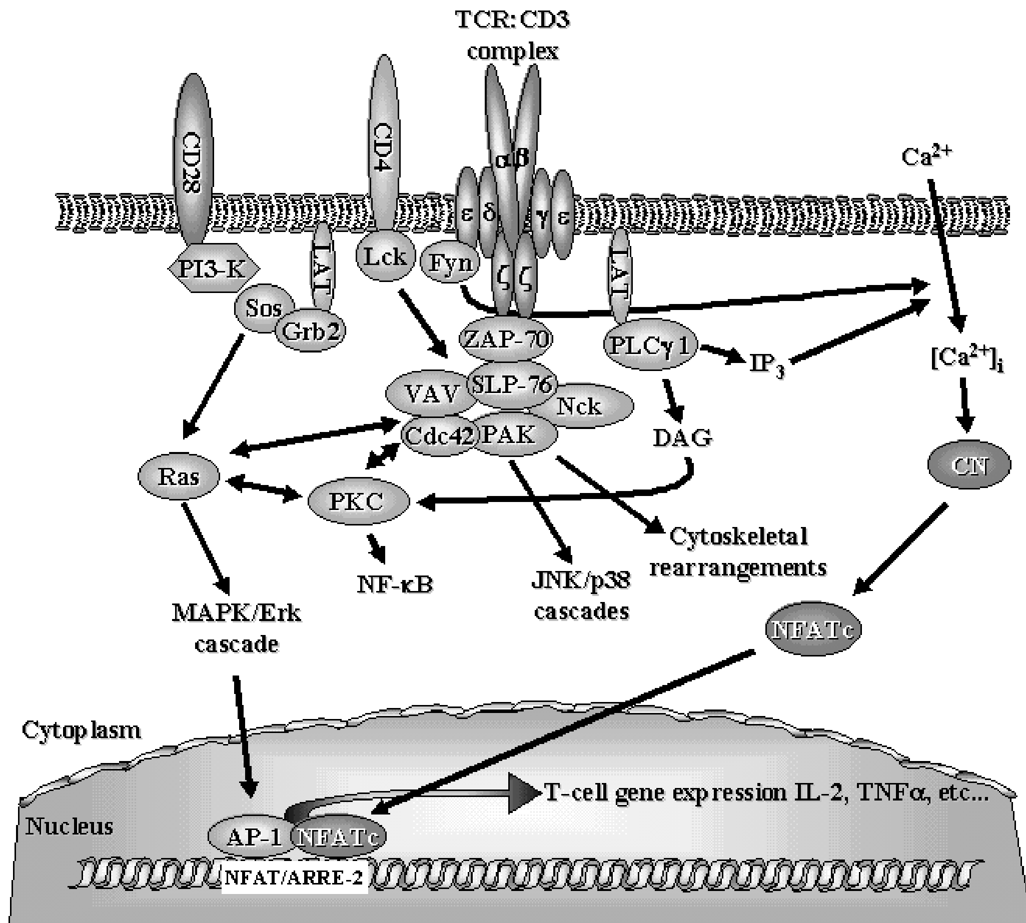

Figure 4. Schematic representation of TCR-triggered activation of NFAT-dependent transcription.

In the nucleus these two important signaling pathways again converge on a family of transcription factors referred to as the Nuclear Factor of Activated T cells (NFAT), which regulates a set of genes important for T cell activation, such as IL-2, IL-4, tumour necrosis factor (TNF)-α and FasL (Shaw *et al.* 1988, Goldfeld *et al.* 1993, Szabo *et al.* 1993, Latinis *et al.* 1997). The NFAT family consists of four "classical" members NFAT1/NFATp/NFATc2, NFAT2/NFATc/NFATc1, NFAT3/NFATc4 and NFAT4/NFATx/NFATc3 (Masuda *et al.* 1998, Crabtree 1999). Recently, a novel member of the NFAT family, NFAT5/TonEBP, was characterized but its biological properties seem to be quite distinct from those of the other four (Lopez-Rodriguez *et al.* 1999, Miyakawa *et al.* 1999). Transcriptional activation of NFAT target genes usually requires coincident activation of NFAT together with certain other transcription factors, most often the activator protein 1 (AP-1) complex. The AP-1 complex, activated via the Ras/MAPK pathway, is a dimer composed of members of the Jun and Fos

families of transcription factors (Crabtree 1999). The cooperative binding of the NFAT and the AP-1 complex to the NFAT-binding sites enhances the stability of NFAT/DNA-interaction (Crabtree 1999).

The activity of the NFAT is regulated by Ca^{2+}-dependent serine/threonine phosphatase, calcineurin, which dephoshorylates NFAT and thereby triggers its nuclear translocation. In order to activate its target genes, NFAT has to be maintained in the nucleus. Since rephosphorylation of NFAT by a constitutive cellular kinase activity results in nuclear export of NFAT, a prolonged Ca^{2+} stimulus is required for effective activation of NFAT (Timmerman *et al.* 1996, Dolmetsch *et al.* 1997). In T cells, such a Ca^{2+} signal is generated by activation of the capacitative calcium entry (CCE) (Putney 1999). Activation of TCR stimulates production of IP_3 (via PLCγ1 activation) that evokes a biphasic increase in intracellular free Ca^{2+} levels. An IP_3-dependent activation of inositol 1,4,5-trisphosphate receptor (IP_3R) in the endoplasmic reticulum (ER) membrane triggers a rapid and transient Ca^{2+} signal by releasing Ca^{2+} from the intracellular Ca^{2+} stores in the ER. Depletion of these stores activates store-operated calcium channels (SOCs) in the plasma membrane by a mechanism that is incompletely understood (Putney 1999). Some investigators believe that a soluble messenger mediates this signal whereas others have suggested that physical connection between the channel molecules, IP_3R and SOC, is required for activation of CCE (Putney 1999).

Effects of Nef on T cell signaling

The effects of Nef on T cell signaling have been extensively studied. However, drawing a coherent picture based on these observations is difficult because not all of the experimental systems are comparable with each other. Productive infection of resting peripheral blood mononuclear cells (PBMCs) requires activation of CD4-positive T lymphocytes (Stevenson *et al.* 1990). Furthermore, HIV-LTR-driven transcription is induced by T cell mitogens (Siekevitz *et al.* 1987, Tong-Starksen *et al.* 1987). Because of such a correlation between T cell activation and viral replication it would seem logical that the effects of Nef on T cell signaling were positive. Accordingly, studies in Nef-transgenic mice demonstrated hypersensitive T cell responses (Skowronski *et al.* 1993, Hanna *et al.* 1998). Surprisingly, several studies in lymphoid cell lines implicate Nef as a negative regulator of T cell signaling (Table 1). Although the proposed mechanisms of how Nef modulates T cell activation are diverse, the T cell receptor (TCR)-triggered cascades, especially the receptor proximal events, have frequently been

suggested to be the targets for Nef function (positive or negative). The possible roles of some of the reported Nef effectors are discussed below.

Table 1. Reported effects of Nef on T cell activation

Negative effects	• Nef blocks TCR- or mitogen-induced production of IL-2 mRNA (Luria *et al.* 1991) • Nef inhibits TCR- or mitogen-induced DNA-binding of NF-κB and AP-1 (Niederman *et al.* 1992, Niederman *et al.* 1993, Bandres and Ratner 1994) • Inhibition or activation of the early events of TCR-signaling by Nef depending on its intracellular localization (Baur *et al.* 1994) • Nef interacts with Lck and inhibits its catalytic activity (Collette *et al.* 1996) • Nef binds to Lck and MAPK and inhibits their catalytic activities (Greenway *et al.* 1996) • Nef inhibits induction of an early activation antigen CD69 by blocking a TCR-proximal event (Iafrate *et al.* 1997)
No effects	• Nef does not alter mitogen- or various receptor-mediated T cell responses (Schwartz *et al.* 1992, Carreer *et al.* 1994) • Expression of Nef from a stably integrated provirus does not modify responses in antigen-dependent T cells upon antigenic stimulus (Page *et al.* 1997) • Nef does not modulate mitogen-induced activation of NF-κB or AP-1 (Yoon and Kim 1999)
Positive effects	• Thymocytes from Nef-transgenic mice are hyperresponsive to TCR-mediated stimulus (Skowronski *et al.* 1993, Hanna *et al.* 1998) • Activation or inhibition of the early events of TCR-signaling by Nef depending on its intracellular localization (Baur *et al.* 1994) • Nef stimulates production of IL-2 from Herpesvirus saimiri transformed T cells (Alexander *et al.* 1997) • Nef from an aggressive strain of SIV (pbj14) activates T cell signaling (Luo and Peterlin 1997) • Nef upregulates FasL expression by interacting with the TCRζ chain (Xu *et al.* 1999) • Nef enhances T cell responsiveness for TCR-mediated activation (Schrager and Marsh 1999, Schibeci *et al.* 2000, Wang *et al.* 2000) • Nef interacts with IP3R1 to activate Ca^{2+}/calcineurin pathway independently of TCR-proximal signaling events (**IV, V, VI**)

Nef and T cell receptor zeta chain (TCRζ)

The Nef proteins of HIV-2 and SIV (but not HIV-1) were shown by two groups to interact with the zeta chain of T cell receptors (TCRζ) (Bell *et al.* 1998, Howe *et al.* 1998). Subsequently, Xu *et al.* demonstrated that a membrane-targeted form of HIV-1 Nef associated with TCRζ but via different molecular interactions (Xu *et al.* 1999). A functional PxxP-motif of HIV-1 Nef was required for TCRζ-binding (Xu *et al.* 1999), whereas abrogating this region in HIV-2 or SIV Nef had little effect on their association with TCRζ (Howe *et al.* 1998). Possible biological functions related to these interactions included downregulation of components of the T cell receptor (Bell *et al.* 1998) and upregulated surface expression of FasL in infected cells (Xu *et al.* 1999). Upregulation of FasL expression by Nef has been reported also in several other studies (Dittmer *et al.* 1995, Xu *et al.* 1997, Hodge *et al.* 1998b, Zauli *et al.* 1999). The increased abundance of this pro-apoptotic ligand for Fas (Apo-1, CD95) on the cell surface may protect infected cells from CTL-mediated killing by inducing apoptosis in Fas-expressing HIV-specific CTLs (Xu *et al.* 1997, Xu *et al.* 1999, Zauli *et al.* 1999).

Nef and Src-family tyrosine kinases

The interactions between the PxxP-motif of Nef and the SH3 domains of Hck and Lyn were the first PxxP-mediated interactions characterized for Nef (Saksela *et al.* 1995). More recently an SH3-mediated binding of other Src family kinases, Lck (Greenway *et al.* 1995, Collette *et al.* 1996, Greenway *et al.* 1996, Dutartre *et al.* 1998, Cheng *et al.* 1999), Fyn (De and Marsh 1994, Lee *et al.* 1995, Arold *et al.* 1997, Arold *et al.* 1998, Cheng *et al.* 1999) and Src (Du *et al.* 1995, Lang *et al.* 1997) to Nef have been reported. The affinity of these interactions, however, seems to be several orders of magnitude lower than those of Nef to Hck or Lyn (Lee *et al.* 1995, Arold *et al.* 1998). The interaction surfaces between the PxxP-motif of Nef and the SH3 domains of Hck or Fyn have been described in detail (Grzesiek *et al.* 1996b, Lee *et al.* 1996, Arold *et al.* 1997, Grzesiek *et al.* 1997). A left-handed polyproline helix formed by the proline-rich sequence is the minimal ligand for SH3 domains, and additional specificity and strength are provided by stabilizing interactions between the SH3 variable loop (RT-loop) and regions in Nef outside of the PxxP-motif (Lee *et al.* 1996).

Nef induces a catalytically active conformation of Hck *in vitro* by binding to its SH3 domain and thereby relieving intramolecular inhibitory interactions (Moarefi *et al.* 1997). Briggs *et al.* reported that such activation also takes place in fibroblasts as co-expression of Nef and Hck led to malignant transformation in Rat1 fibroblasts (Briggs *et al.* 1997). Also in monocyte/macrophage-like cells, which are a more relevant model for studying Hck activation, Nef and Hck have been shown to interact with each other (Foti *et al.* 1999). Moreover, Biggs *et al.* observed Hck-dependent induction of AP-1 DNA-binding in Nef-expressing macrophages (Biggs *et al.* 1999). In addition to the enhanced transcription of the integrated provirus, Nef-mediated activation of Hck in infected monocytes/macrophages could lead to stimulated secretion of cytokines and/or other factors that establish a favourable environment for virus spread as reported by Swingler *et al.* (Swingler *et al.* 1999, see page 27).

The expression pattern of Hck, as well as the lesser-studied Lyn that is likely to be analogously regulated by Nef, suggests that upregulation of its catalytic activity may play a role only in a subset of HIV-susceptible cells, namely macrophages and monocytes (Brickell 1992). However, most of the HIV replication takes place in CD4-positive T lymphocytes that predominantly express Lck and Fyn (Brickell 1992). Lck and Fyn are intimately involved in the early events of T cell receptor-mediated signaling, where they have partially redundant roles (Denny *et al.* 2000). In contrast to Hck, Lck activity seems to be negatively regulated by Nef (Collette *et al.* 1996, Greenway *et al.* 1996). Whether Nef has any effects on the activity of Fyn in T cells is not known, but in Rat2 fibroblasts co-expression of Nef inhibits the transforming activity of Fyn (Briggs *et al.* 2000). It is not clear how inhibition of the catalytic activities of Lck and/or Fyn in T cells could contribute to the positive effects of Nef on viral replication. In any case, the molecular mechanism must be fundamentally different from that suggested for Nef and Hck in monocytes/macrophages.

An uncharacterized serine kinase reportedly associates with the N-terminus of Nef (Baur *et al.* 1997). The biological relevance of this interaction is not known but it may stabilize the association between Nef and Lck (Baur *et al.* 1997).

Nef and VAV

Fackler *et al.* implicated VAV, a guanine nucleotide exchange factor centrally involved in T cell signaling, as a Nef effector molecule (Fackler *et al.* 1999). Nef was found to bind via its proline-rich region to the C-terminal SH3 domain of

VAV1 and VAV2 (Fackler *et al.* 1999). This led to cytoskeletal rearrangements and activation of the JNK/p38-cascade in murine fibroblasts (Fackler *et al.* 1999). Although it is not known whether the activation of VAV by Nef occurs in T cells, this observation represents another possible mechanism how Nef may promote viral replication by activating T cells.

Nef and p21-activated kinase 2 (PAK2)

Sawai *et al.* reported that Nef associates with a cellular 62kDa serine threonine kinase, which they called Nef-associated Kinase (NAK) (Sawai *et al.* 1994). Subsequently, accumulating evidence suggested that this kinase belongs to the family of p21-activated kinases (PAKs) (Lu *et al.* 1996, Nunn and Marsh 1996, Sawai *et al.* 1996, Sawai *et al.* 1997, **I**). Recently, we identified this kinase as PAK2 (**II**). This finding was confirmed by others (Arora *et al.* 2000) while Fackler *et al.* suggested that also PAK1 could also act as NAK (Fackler *et al.* 2000).

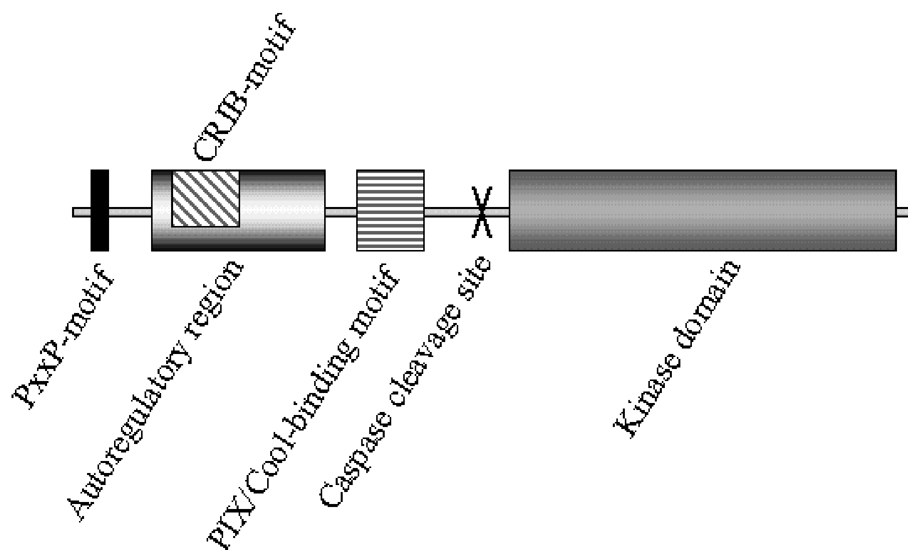

Figure 5. Schematic representation of human PAK2

PAKs have been implicated in a variety of cellular responses, reorganization of the actin cytoskeleton, activation of the MAPK-signaling cascades and apoptosis (Sells and Chernoff 1997, Bagrodia and Cerione 1999). Interestingly, PAK signaling seems to play an important role also during T cell activation (Bubeck Wardenburg *et al.* 1998, Yablonski *et al.* 1998). Four mammalian PAK family members have been identified, PAK1 (PAKα), PAK2 (PAKγ), PAK3 (PAKβ),

which are highly homologous and PAK4 that is less conserved (Bagrodia and Cerione 1999). PAK1, -2 and -3 contain, in addition to nearly identical C-terminal kinase domain, several conserved motifs known to mediate protein-protein interactions. The N-terminal PxxP-motif binds to the second (of three) SH3-domain of an adaptor protein Nck (Bagrodia *et al.* 1995b, Lu *et al.* 1997, Zhao *et al.* 2000). The autoregulatory (AR) region consisting of a Cdc42/Rac1 interactive binding (CRIB)-domain and an autoinhibitory domain is also located in the N-terminus (Burbelo *et al.* 1995, Thompson *et al.* 1998, Lei *et al.* 2000, Morreale *et al.* 2000). The PIX/Cool-binding motif preceding the kinase domain connects PAKs with the guanine exchange factor (GEF) β-PIX/Cool-1 (Bagrodia *et al.* 1998, Manser *et al.* 1998). Interestingly, only PAK2 has a recognition site for DEVD-sensitive caspases (Rudel and Bokoch 1997). The caspase-mediated proteolytic cleavage generates a constitutively active form of PAK2 that seems to be involved in apoptotic processes (Rudel and Bokoch 1997, Rudel *et al.* 1998).

The catalytic activities of the three highly homologous PAKs (1-3) are controlled at multiple levels. In the absence of a bound G-protein, the catalytic domain of PAK interacts with the autoinhibitory domain adjacent to the CRIB-motif thereby locking the enzyme in an inactive conformation (Frost *et al.* 1998, Zhao *et al.* 1998, Tu and Wigler 1999). Upon CRIB-mediated binding of PAK to the GTP-bound Cdc42 or Rac1, the intramolecular inhibitory interaction is disrupted resulting in an active conformation of PAK (Manser *et al.* 1994, Martin *et al.* 1995). Another level of control is brought about by regulation of the cellular localization of PAK by intermolecular interactions. The PIX/Cool proteins appear to recruit PAK to the focal complexes (Manser *et al.* 1998). Although the PIX/Cool-mediated targeting of PAKs to these complexes is important for PAK-induced changes in the cytoskeletal organization, such as formation of membrane ruffles, lamellipodia, filopodia and focal complexes, the exact role of PAK in these phenomena has remained somewhat elusive because of the incoherence of the reported findings (Bagrodia and Cerione 1999). A partial explanation for these differences could be that PAK seems to regulate morphological changes by both kinase-dependent and -independent mechanisms (Frost *et al.* 1998). Engagement of receptor tyrosine kinases (RPTKs) or TCR leads to plasma membrane targeting of the adaptor protein Nck that recruits PAK by binding to its N-terminal PxxP-motif (Galisteo *et al.* 1996, Lu *et al.* 1997, Yablonski *et al.* 1998). Membrane-recruitment seems to be sufficient for PAK activation that is likely to be mediated by Cdc42/Rac1-dependent mechanisms (Lu *et al.* 1997). PAKs have been implicated in activation of the MAPK subgroups JNK/p38 (Bagrodia *et al.* 1995a, Zhang *et al.* 1995, Brown *et al.* 1996) and Erk2 (Lu *et al.* 1997, King *et al.* 1998, Yablonski *et al.* 1998). Whereas a functional interplay between PAK and Raf-1 at least partially mediates the Erk2 activating effects of PAK (King *et al.* 1998), the mechanism by which PAK regulates the JNK/p38-activity is less clear.

Multiple regions in Nef have been shown to be important for association with PAK2. Although PAK2 does not contain SH3 domains, the PxxP-motif of Nef is critical for its ability to coprecipitate with PAK2 (Wiskerchen and Cheng-Mayer 1996, Khan *et al.* 1998, **I**). Therefore, it is possible that another protein containing an SH3 domain is involved in forming a trimeric complex with Nef and PAK2. Residues outside the proline-rich region of Nef have also been implicated in PAK2 interaction (Sawai *et al.* 1995, Wiskerchen and Cheng-Mayer 1996, **I**).

The ability of Nef to associate with PAK2 has been correlated with enhanced viral replication, particle infectivity and induction of simian AIDS (Wiskerchen and Cheng-Mayer 1996, Khan *et al.* 1998). In contrast, Luo *et al.* found that the enhancement of HIV infectivity by Nef is independent of PAK association, at least for some viral isolates (Luo *et al.* 1997). Moreover, Lang *et al.* observed that macaques infected with a mutant virus developed simian AIDS before significant amount of reversions to the PAK-binding phenotype emerged (Lang *et al.* 1997). Whether the capacity to bind to PAK2 represents a biologically significant function of Nef, remains to be proven.

Nef and protein kinase C-θ (PKCθ)

Protein Kinase C isozyme theta (PKCθ) is the only PKC isozyme that is recruited to the supramolecular activation clusters (SMACs) upon TCR activation (Monks *et al.* 1997). Moreover, several lines of evidence suggest an important role for PCKθ in T cell activation (Werlen *et al.* 1998, Villalba *et al.* 2000). Nef has been reported to coprecipitate with PKCθ (Smith *et al.* 1996). Upon activation (e.g. with mitogens), PKC isoforms translocate from the soluble fraction to the particulate fraction. In Nef-expressing cells, such a translocation of PKCθ did not occur (Smith *et al.* 1996). Moreover, the levels of PKCθ were reduced in Nef-expressing cells, and the authors speculated that Nef inhibits binding of PKCθ to its natural substrates and thereby renders it susceptible for degradation. These effects of Nef on PKCθ may lead to impaired signaling in T cells (Smith *et al.* 1996).

Nef and c-Raf1

The c-Raf-1 serine/threonine protein kinase is an integral part of the Ras/MAPK cascade that plays a critical role in the proliferation of most cell types, including T cells (Yuryev and Wennogle 1998). A highly conserved acidic carboxy-terminal region in Nef was shown to mediate binding of Nef to c-Raf1 (Hodge *et al.* 1998a). Interestingly, mutating this motif abrogated the ability of Nef to downregulate CD4 expression (Aiken *et al.* 1996, Iafrate *et al.* 1997). These same residues have also been implicated in binding of Nef to the catalytic subunit of vacuolar ATPase (Lu *et al.* 1998).

Nef and mitogen activated protein kinase p44 (MAPK/Erk1)

Greenway *et al.* demonstrated that the mitogen activated protein kinase p44 (MAPK/Erk1) could be coprecipitated from cellular extracts with a recombinant glutathione-S transferase (GST)-Nef fusion protein (Greenway *et al.* 1995). The binding of Nef to Erk1 required the PxxP-motif of Nef and led to inhibition of the catalytic activity of Erk1 (Greenway *et al.* 1996). It is unclear how the SH3-ligand domain of Nef contributes to this interaction because Erk1 does not have SH3 domains.

Other mechanisms of modulation of T cell signaling by Nef

Swingler *et al.* recently reported that Nef-expression in macrophages induced secretion of two chemokines, macrophage inflammatory protein (MIP)-1α and MIP-1β and a yet uncharacterized T cell activating protein (Swingler *et al.* 1999). Via recruitment and activation of T lymphocytes, these factors could provide new susceptible target cells at sites of viral replication (Swingler *et al.* 1999). Perhaps related to this finding, Nef has been shown to upregulate activator protein 1 (AP-1)-mediated signaling (Biggs *et al.* 1999) and to modulate calcium metabolism (Foti *et al.* 1999) in macrophage/monomyelocytic cell lines. Another interesting possibility is raised by studies suggesting that also extracellular Nef protein could contribute to the deregulation of cellular signaling in T cells and/or monocytes/macrophages (Brigino *et al.* 1997, Alessandrini *et al.* 2000, Haraguchi *et al.* 2001).

5. AIMS OF THE STUDY

Nef has been shown to modulate cellular signal transduction pathways, but the molecular mechanism of this function of Nef as well as the critical cellular Nef effector molecules have remained elusive. The aim of this study was to develop *in vitro* models and use them to study the effects of Nef expression on cellular signal transduction pathways and to characterize cellular proteins interacting with Nef.

Detailed aims of the study were:

1. To characterize the Nef-associated kinase (NAK) and the structural/ functional requirements for Nef/NAK interaction

2. To study the effects of Nef expression on T cell activation and characterize the molecular mechanisms of these effects

6. MATERIALS AND METHODS

6.1. Cell lines

The human T cell leukaemia cell line Jurkat E-6 (JE-6) derived from American Type Culture Collection (ATCC; Bethesda, MD), J.CaM1.6 (a Jurkat clone devoid of functional Lck expression provided by Dr. Tomas Mustelin), J.51-31 (a Jurkat clone stably expressing inducible BH10 Nef construct) and its parental clone MT11 (Cooke *et al.* 1997) (both kindly provided by Dr. Mark Harris, Leeds Univ., UK), A3.01 (a CEM, human T cell line derivative obtained from National Institutes of Health AIDS Research and Reference Reagent Program), a Jurkat subclone stably expressing the antisense cDNA for IP_3R1 (Jayaraman *et al.* 1995), referred to as J.IP3R1AS later in this study (kindly provided by Dr. A. Marks, Columbia University, New York) and MT-4 T cells (a kind gift from Anssi Lagerstedt from our institute) were grown in RPMI 1640 (BioWhittaker) medium supplemented with 2mM glutamine (Hyclone), 10% fetal bovine serum and with or without antibiotics (100units/ml penicillin and streptomycin; Gibco, Lifetechnologies) as indicated. HEK293T cells, a human embryonic kidney fibroblast-derived cell line (ATCC), HepG2, a human hepatoma cell line (ATCC) and HeLa (BH10) cell line stably transfected with inducible BH10 Nef construct (Cooke *et al.* 1997) were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) containing 10% FCS, 2mM L-glutamine, and 100units/ml of penicillin and streptomycin (Gibco, Lifetechnologies).

6.2. Antibodies

Polyclonal αCD3 (HIT3a) was purchased from Pharmingen (San Diego, CA). Polyclonal sheep-αNef (BH10)/αGST-antibody was kindly provided by Dr. Mark Harris (Leeds University, UK). Mouse monoclonal antibodies (2A3, 3A2, 6.2, 2H12, 3E6, 3F2.1, 3D12 and 2F2) raised against different epitopes of Bru isolate of HIV-1 Nef were provided by Dr. K. Krohn from our institute. Fluorescein isothiocyanate (FITC)-conjugated CD4-antibody was purchased from Pharmingen. Anti-PAK antibodies (P1N, P1C, P2N, PAK2V and P3N) were purchased from Santa Cruz Biotechnology. Antibodies against PAK1-R1, PAK2-R2 and PAK3-R3 were raised in rabbits against cocktails of selected sequences that are divergent in the different PAK proteins. Peptides were made as multiple-antigen peptides, $(peptide)_8$-K_4-K_2-K-A. Anti-HA antibodies were from BabCO.

6.3. Transfection

Calcium phosphate (I)

Plasmid DNA was mixed with 62µl of 2M $CaCl_2$ and sterile water was added up to 500 µl. An equal volume of 2×HEPES solution (280mM NaCl, 1.5mM Na_2HPO_4, 55mM N-2-hydroxyethylpiperazine-N´-2-ethanosulfonic acid (HEPES) pH 7.0) was added dropwise into the DNA/$CaCl_2$ mixture while tapping the tube. Transfection mixture was immediately dispersed onto exponentially growing 293T cells (~ 50-70 % confluency) in a 10-cm dish (Greiner). The next day the medium was replaced with fresh medium.

Lipofectamine (II, IV)

Lipofectamine (Gibco Life Technologies) transfections of 293T cells were carried out in 6-well plates (Greiner) according to the manufacturer's instructions. In brief, 24 hours before transfections, 6×10^5 cells were seeded per well. A total of 2µg of plasmid DNA was diluted by 100µl of OPTIMEM (Gibco Life Technologies) and mixed with 5µl of Lipofectamine in 895µl of OPTIMEM. The mixture was incubated for 30 minutes at room temperature, after which it was added dropwize onto the cells. After 4 hours of incubation (+37°C, 5% CO_2), 2ml of complete DMEM was added to the wells.

Fugene (III, IV)

Jurkat E-6 cells (JE-6; from ATCC) were maintained in complete RPMI 1640 medium without antibiotics. The cultures were diluted to 5-6×10^5 cells per millilitre one day prior to transfection. Two million exponentially growing cells were transfected with Fugene transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. In short, Fugene (3:2 ratio {µl Fugene/µg DNA}; total amount of DNA ~ 4µg/2 million cells) was incubated with OPTIMEM medium (Gibco, Life Technologies) for 5 minutes at RT before adding the mixture onto undiluted DNA in an eppendorf tube. The Fugene/DNA mixture was incubated for a further 15-30 minutes at RT followed by dropwize pipetting onto cells.

DMRIE-C (IV, V, VI)

Jurkat E-6, JIP_3R1AS, A3.01 and MT-4 cells were maintained in complete RPMI 1640 (BioWhittaker) medium. DMRIE-C (5:6 ratio {µl DMRIE-C/µg DNA}: total amount of DNA ~ 4µg/1.5million cells) was incubated in 250µl of OPTIMEM for 20 minutes at room temperature. Plasmid DNA was diluted in 250µl of OPTIMEM. DMRIE-C and DNA dilutions were mixed and incubated at RT for 45 minutes. Exponentially growing cells were pelleted, washed once with OPTIMEM, resuspended into OPTIMEM at 1.5×10^6 cells/ml and pipetted onto lipid/DNA mixture. The cells were incubated in a humidified incubator (37°C, 5% CO_2) for 4 hours, and 1.5ml of RPMI 1640 supplemented with 2mM glutamine and 15% FCS per well was added.

6.4. Measurement of protein concentration (I, II, III)

The protein concentrations of cytoplasmic cell extracts were measured with Biorad protein assay kit (Biorad) according to the manufacturer's instructions. In brief, 12µl of the lysate was mixed with 62µl of the A+S solution in a 1cm light path cuvette (Kartell). Five hundred microliters of solution B was added into the cuvette followed by immediate gentle vortexing. After 15-30 minutes incubation absorbance was read at 750nm wavelength with a Genesys 5 spectrophotometer (Spectronic).

6.5. Immunoprecipitation (I, II, III, IV)

The cells were washed once with standard phosphate-buffered saline and lysates were prepared using either *In Vitro* Kinase assay (IVKA) lysis buffer (50mM N-2-hydroxyethylpiperazine-N′-2-ethanosulfonic acid (HEPES) pH 7.4; 150mM NaCl; 10% glycerol; 1% Triton X-100; 1mM ethyleneglycol-bis-β-aminoethyl ether N,N,N',N' tetra-acetic acid (EGTA); 1.5mM $MgCl_2$, containing 1mM phenylmethylsulfonylfluoride (PMSF), 10µg/ml aprotinin, 10mM sodium fluoride and 1mM sodium orthovanadate) (**I, II, VI**) or 1×Cell Culture Lysis buffer (Promega) (**IV**). Appropriate antibodies were added to the cell lysates followed by a 4 hours to overnight agitated incubation at +4°C. Protein A (or G) Sepharose beads (Sigma) were added and the incubation was continued for 30 minutes. The beads were pelleted (table-top centrifuge, 5000 rpm, 30 seconds), washed three times with 500µl of IVKA lysis buffer, with a centrifugation step in between each successive wash.

6.6. Western blotting (I, II, III, V, VI)

Immunocomplexes (from immunoprecipitation) were boiled in ~ 30 µl of 1×Laemmli sample buffer (200mM Tris-HCl pH8.8, 20% glycerol, 5mM ethylenediamine N,N,N',N' tetraacetic acid (EDTA), 0.02% bromophenol blue (BPB), 4% sodium dodecylsulphate (SDS), 50mM dithiotreitol (DTT)). Whole cell extracts and cytoplasmic extracts were mixed with an equal volume of 2×Laemmli sample buffer. Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filters

(Hybond) with a semi-dry blotter (Biorad) according to standard protocols. Filters were rinsed once in standard phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T 0.05%) and blocked either in PBS-T 0.05% containing 5% bovine serum albumin (BSA) and 0.05% NaN_3 or in PBS-T 0.05% containing 5% non-fat milk at RT for 45 minutes or overnight at +4°C. The blocking solution was removed and a primary antibody was added in PBS-T 0.05% followed by one hour (RT) to overnight (+4°C) incubation. After three washes with PBS-T 0.05%, the membranes were incubated for 30 minutes with a 1:3000 dilution of biotinylated αmouse- or αrabbit-IgG in PBS-T 0.05% containing 3% BSA and 0.05% NaN_3, washed three times with PBS-T 0.05%, incubated 20 minutes with a 1:5000 dilution of streptavidin-conjugated horseradish peroxidase (Amersham) in PBS-T 0.05% and washed again three times. Antibody/enzyme-complexes were visualized by enhanced chemiluminescence (ECL) (Amersham) according to the manufacturer's instructions.

6.7. *In vitro* kinase assay (I, II, III)

The immunoprecipitated complexes on beads were subjected to two additional washes with IVKA buffer (50mM HEPES, pH 7.4; 10mM $MgCl_2$). γ-^{32}P-ATP (2,5μCi) was added to the bead-bound immunocomplexes (in 50-100 μl of IVKA buffer) and incubated at +37 C for 20 minutes. The beads were washed twice with ice-cold PBS and boiled for 2 minutes in 1×Laemmli sample buffer. The phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography.

6.8. Re-immunoprecipitation assay (II, III)

After immunoprecipitations and subsequent *in vitro* kinase assays, the beads were washed with PBS and incubated with 1mg/ml of the peptide corresponding to the epitope used for immunoprecipitations. Eluted proteins were subjected to beads prebound with antibodies and incubated overnight at +4°C. The beads were washed with PBS and boiled in 1×Laemmli sample buffer. Proteins were run on SDS-PAGE and visualized by autoradiography.

6.9. Limited in-gel protease digestion (II)

Radiolabelled bands were cut from SDS-polyacrylamide gels and allowed to rehydrate in a 1:10 diluted Laemmli sample buffer. Gel pieces were transferred into the wells of a 13 % SDS-polyacrylamide gel and minced with a needle. Chymotrypsin (500ng per well, Sigma) or endoproteinase Glu-C from *Staphylococcus Aureus* V8 (2μg per well, Sigma) were added into the 1:10 Laemmli sample buffer. After stacking, the electrophoresis was stopped for 30 minutes and then continued overnight. After the electrophoresis, the gel was dried and exposed to Biomax MR film (Kodak).

6.10. *In vitro* caspase digestion (II)

After the *in vitro* kinase assay, the beads were washed with PBS, equilibrated with digestion buffer (DB; 25mM HEPES pH7.4, 1mM DTT) and incubated with 40ng of caspase 3 (Calbiochem) in DB at +37°C for the indicated time points. The reactions were stopped by the addition of 1×Laemmli sample buffer.

6.11. Luciferase reporter assay (IV, V)

Twenty hours after transfection the cells were either left untreated or were treated with various agents (50ng/ml αCD3, 1μg/ml αCD28, 50 ng/ml PMA, 1μg/ml A23187, 30ng/ml thapsigargin) or their combinations for 4-6 hours prior to their harvest and lysis. When indicated, cells were pretreated for 30 minutes before stimulations with one of the following substances: 200nM cyclosporin A (CsA), 500μM EGTA, 200μM wortmannin (WM), 5nM 4-amino-5-(4-methylphenyl)-7-(t-butyl)-pyrazolo-[3,4-d]-pyrimidine (PP1) or 75nM 2-aminoethyoxydiphenyl

borane (2-APB). Cells were pelleted and washed once with standard phosphate buffered saline and lysed in 200µl of cell culture lysis buffer (Promega, Madison, WI). Luciferase activity was measured with Promega luciferase reagents and a Luminova 1254 (Labsystems, Finland) luminometer. For the measurement of β-galactosidase activity 100µl of lysate was mixed with 10µl of LacZ-buffer (500nM NaCl, 100nM $MgCl_2$, 100mM β-mercaptoethanol) followed by addition of 100µl of 10mM O-nitrophenyl-β-D-galactopyranoside (ONPG; from Sigma). The reactions were incubated at +37°C overnight or until yellow colour appeared, after which their absorbances were measured at 420 nm. The absorbance value of each sample was divided by the mean of all the samples. The raw luciferase counts of each sample were divided by this ratio to normalize for transfection efficiency.

6.12. Assay for analyzing CD4-downregulation (V)

Forty-eight hours after transfection the cells (cotransfected with either empty pEF-BOS expression vector or different Nef mutants together with pEF-BOS-GFP and pSV40-CD4) were harvested and washed with PBS containing 0,1 % BSA (PBS-B). The cells were labeled with 10µl phycoerythrin-conjugated anti-human CD4-antibody (Pharmingen) for 30 minutes. After two washes with PBS-B, the labeled cells were fixed with PBS containing 1% paraformaldehyde. The surface expression of CD4 was analyzed on GFP-positive cells by FACScan flow cytometry (Becton Dickinson).

6.13. Recombinant protein production in *Escherichia coli* (I)

The expression and purification of the GST and MBP fusion proteins in *Escherichia coli* BL21 were carried out according to manufacturer's instructions (Pharmacia and New England Biolabs, respectively). After elution from their respective affinity beads, the fusion proteins were concentrated and changed into the desired buffers by successive rounds of micro concentration using Centrex UF2 columns (Schleicher & Schuell). The buffer of the GST-Nef proteins was changed to HBS (10mM HEPES pH7.4, 150mM NaCl, 3mM EDTA) containing 0.005% (v/v) surfactant P20 and 1mM dithiotreitol (DTT), (HBS-plus) in which they were subjected to thrombin cleavage (1U/0.2mg fusion protein) for 4-6 hours at +37°C. The MBP proteins were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin reagent as suggested by the manufacturer (Pierce), but less biotin was used, molar ratio of protein:biotin was 1:2). The samples were then subjected to multiple additional rounds of microconcentration to remove any free biotin.

6.14. Measurement of protein-protein interactions by surface plasmon resonance (I)

Surface plasmon resonance (SPR) experiments were carried out using Biacore X apparatus (Pharmacia Biosensor, Uppsala). An SA biosensor chip (Pharmacia Biosensor) with pre-immobilized streptavidin was coated with biotinylated MBP (to the reference channel) or MBP-Hck-SH3 (to the test channel) proteins (100ng/ml in HBS-plus) by serial short injections (at a flow rate of 5µl/minute). The attachment of the ligands was monitored by the changes in the refractive index and was set to ~2200 (MBP-Hck-SH3) and ~2000 (MBP) response units (RU), corresponding to the relative difference in their molecular weights. After the immobilization of the biotinylated ligands, the chip was subjected to three rounds of preregeneration cycles, which were subsequently applied once between each Nef injection. One regeneration cycle consisted of successive 1-min pulses (flow rate of 5µl/min) of pH 2.2 glycine buffer, 0.05 % SDS and 4 M urea. Some loss of the refractive index was observed during the first and the second cycles but no longer during the third cycle of this regeneration treatment. The injections of different Nef proteins were done using concentrations ranging from 4.0 to 0.0125 µM, with a flow rate of 5 µl/min at +25°C in HBS-plus buffer. Each chip was used for approximately 50 Nef injections, during which no loss of the immobilized ligand or the capacity of the chip to bind a standard solution of R71 Nef was observed. The sensograms, in which the refractive index values from the reference channel were subtracted (to give corrected resonance units {cRU}), were analyzed

using BIAevaluation (v3.0) software (Pharmacia Biosensor). The Scatchard plots and line fitting was done with Excel (Microsoft) and were based on values from the sensograms at a 20-min post-injection time point.

6.15. Nef/IP_3R1-coimmunoprecipitation assay (VI)

Twenty-four million Jurkat or J.IP3R1 cells were transfected with 25µg of Nef or an empty pEF-BOS vector using DMRIE-C (Gibco, Life Technologies) as described above. Twenty hours later, the cells were washed once in standard phosphate-buffered saline and lysed in 700µl of IVKA lysis buffer. Five hundred microliters of this lysate was mixed with 2µl of the polyclonal rabbit-αIP_3R1-antisera (A.G. Scientific Inc.) and into the remaining 200µl of the lysate 3µl of the sheep-αNef/αGST polyclonal antisera was added. The mixtures were incubated at +4°C for 2 hours. Twenty-five microliters of Protein A-Sepharose beads (1:1 suspension in IVKA lysis buffer) were added and the incubation was continued for 30 minutes at +4°C. The beads were washed three times with 500µl of IVKA, boiled in 1×Laemmli sample buffer and proteins were separated by SDS-PAGE. The proteins were transferred onto a nitrocellulose filter and subjected to western blotting with the mixture of monoclonal αNef-antibodies followed by ECL detection as described above.

6.16. Measurement of intracellular calcium with recombinant aequorin (VI)

Three million Jurkat cells were transfected with 3 µg of pEF-BOS-HA-aequorin (the recombinant aequorin cDNA was derived from pcDNA-HA-aequorin, Molecular Probes) together with other plasmids (total DNA amount up to 6 µg) by using DMRIE-C as described above. Twenty hours later cells were pelleted and resuspended into 500µl of complete RPMI-1640 medium containing 2.5µM coelenterazine h (Molecular Probes). The cells were incubated with coelenterazine h for 1-4 hours, washed twice with 800µl of RPMI-1640 containing 2mM glutamine and 1% FCS but without phenol red. The cells were suspended into 300µl of this medium and calcium-dependent light production was continuously monitored with a Luminova 1254 (Labsystems, Finland) luminometer. At the end of each experiment, the cells were lysed in 300µl of hypotonic lysis buffer (10 mM Tris-HCl pH7.2, 0.1 mM EGTA) followed by addition of 30mM $CaCl_2$ to consume the remaining aequorin from each transfection. The total aequorin signal measured along the whole experiment was used to normalize for transfection efficiency.

6.17. Fluorescent confocal microscopy analysis of the GFP-tagged NFATc

One million Jurkat cells were transfected with 2µg of pCMV-GFPNFATc (a kind gift from Päivi Koskinen, University of Turku) together with 1µg of pEBB-Nef or an empty pEF-BOS expression vector with DMRIE-C as described above. Twenty hours later cells were spun down and resuspended into 30µl of RPMI-1640 complete medium. An equal volume of prewarmed (40°C) PBS containing 1% low melting point agarose (molecular biology grade, FMC) was added and the mixture was immediately pipetted between a glass slide and a coverslip (separated by thin plastic spacers). Fluorescence microscopy images were taken with an Ultraview confocal imaging system (Perkin Elmer) using Olympus X70 microscope.

7. RESULTS AND DISCUSSION

7.1. Characterization of NAK as PAK2 (I, II, III)

Recent studies have indicated that Nef-associated kinase (NAK) has several properties resembling those of the p21-activated kinases (PAKs) (Lu *et al.* 1996, Nunn and Marsh 1996, Sawai *et al.* 1996). Four PAK isoforms have been characterized: PAK1, PAK2, PAK3 and PAK4 (Bagrodia and Cerione 1999). PAK1, 2 and 3 are highly homologous while PAK4 is more divergent and lacks an aminoterminal PxxP-motif that has been shown to bind to Nck (Bokoch *et al.* 1996, Lu *et al.* 1997). Because NAK was found to associate with Nck (**I**, see below), PAK4 was not a probable candidate for NAK.

We found that NAK was activated by a myristylated-Nck-SH3-2 (myrNck-SH3-2) construct (**I**) that has been shown to activate PAK (Lu *et al.* 1997). Moreover, we could confirm that a constitutively active form of a small p21GTPase Cdc42 ($Cdc42^{V12}$), implicated in activation of PAK (Bagrodia and Cerione 1999), robustly activated NAK as reported by others (**I**, Lu *et al.* 1996).

We used immunological and protease digestion-based methods to characterize whether NAK represents a specific PAK-isoform or whether several members of the PAK family can serve as NAK. We found that NAK was immunologically related to PAK2 but not to PAK1 or PAK3 (**II**). The proteolytic digestion patterns of PAK2 and NAK were seemingly identical and clearly differed from those of PAK1 and PAK3 (**II**). Furthermore, NAK, like PAK2, was efficiently cleaved by caspase 3 (**II**, Cohen 1997).

One of the problems in characterization of NAK has been that, although readily visible in autoradiograms, NAK cannot be seen by western blotting techniques. Surprisingly, having identified NAK as PAK2, overexpression of PAK2 did not increase the amount of PAK2 coprecipitating with Nef (**II**). We therefore examined if exogenous PAK2 can, however, replace endogenous NAK in the complex with Nef. Our results clearly showed that exogenous PAK2, but not PAK1, could substitute for endogenous NAK (**II**).

Fackler *et al.* suggested that a Nef allele from the SF2 strain of HIV-1 interacted with PAK1 (Fackler *et al.* 2000). To test if different Nef proteins bind to different PAK isoforms, we analysed different Nef variants (NL4-3, SF2 and HAN-2) in a re-immunoprecipitation assay using our isoform specific anti-PAK antisera (**III**).

Our results showed that regardless of the HIV-1 Nef variant used, NAK was PAK2. Fackler *et al.* used a commercial αPAK1 antiserum that we have shown to recognize both PAK1 and PAK2 (Fackler *et al.* 2000). Moreover, the PAK1-derived peptide used by Fackler *et al.* to inhibit NAK-activity overlaps with the conserved PAK autoregulatory domain and is expected to inhibit the CRIB-mediated activation of all PAKs (Zhao *et al.* 1998, Tu and Wigler 1999, Lei *et al.* 2000). Therefore, the conclusion by Fackler *et al.* that SF2 Nef associates with PAK1 seems to be a misinterpretation caused by reagents that fail to distinguish PAK1 from PAK2. Furthermore, we demonstrated that NAK specifically associates with PAK2 also in different cell lines (**II**). Recently, others have confirmed our conclusion (Arora *et al.* 2000).

7.1.1. Structural features of Nef/PAK2 interaction (I, III)

PAK1 and PAK2 are highly homologous proteins but yet only PAK2 can associate with Nef. To better understand the Nef/PAK2-interaction we pursued a study to characterize the structural/functional determinants of both Nef and PAK2 required for their association.

The SH3 domain-binding function of HIV-1 Nef is required for association with PAK2 (I)

None of the known PAK isoforms contain SH3 domains. Nevertheless, previous studies suggested a role for the SH3-ligand domain of Nef in association with NAK (Wiskerchen and Cheng-Mayer 1996, Lang *et al.* 1997). To study this requirement in more detail, we examined the role of the SH3-binding function of NL4-3 Nef in association with NAK by a mutational approach. As a model for PxxP/SH3-mediated interaction we used a well-characterized interaction between Nef and an SH3 domain of Hck (Lee *et al.* 1995, Lee *et al.* 1996).

Point mutations in the Nef gene were designed, based primarily on the previously published Nef/SH3 cocrystal (Lee *et al.* 1996) and nuclear magnetic resonance (NMR) structures (Grzesiek *et al.* 1997). The core of the PxxP-motif of NL4-3 Nef consists of $P^{72}Q^{73}V^{74}P^{75}L^{76}R^{77}$ residues that correspond to an SH3-ligand consensus sequence (PxϕPXR, where ϕ is a (typically small) hydrophobic amino acid, see figure 6A) (Ren *et al.* 1993, Lim 1996, Mayer and Gupta 1998). The P72 and P75 residues are the PxxP-defining prolines important for the correct

folding of the polyproline type II helix (PPII helix). The V74 and R77 residues of Nef form intermolecular (with an SH3 domain) and intramolecular (within Nef) interactions that not only provide stability but also govern the minus orientation of the PxxP-motif of Nef in binding to an SH3 domain (*i.e.* the PxxP-motifs can bind to SH3 domains in two orientations, N- to C-terminal direction (+) and C-to N-terminal direction (-)) (Feng *et al.* 1994, Lim *et al.* 1994, Lee *et al.* 1996). Due to the helical structure of the PxxP-motif, the side chains of the Q73 and L76 residues point out of the helix on the opposite side as the SH3-contacting residues and are thus not likely to directly participate in formation of the binding surface for an SH3 domain (Figure 6A). In addition to the core PxxP-motif, a hydrophobic pocket located outside the PxxP region provides stability and, more importantly, specificity for Nef/SH3 interaction by accommodating an isoleucine residue on the RT-loop of Hck-SH3 domain (Figure 6B). A phenylalanine residue in Nef at position 90 in part contributes to this hydrophobic pocket.

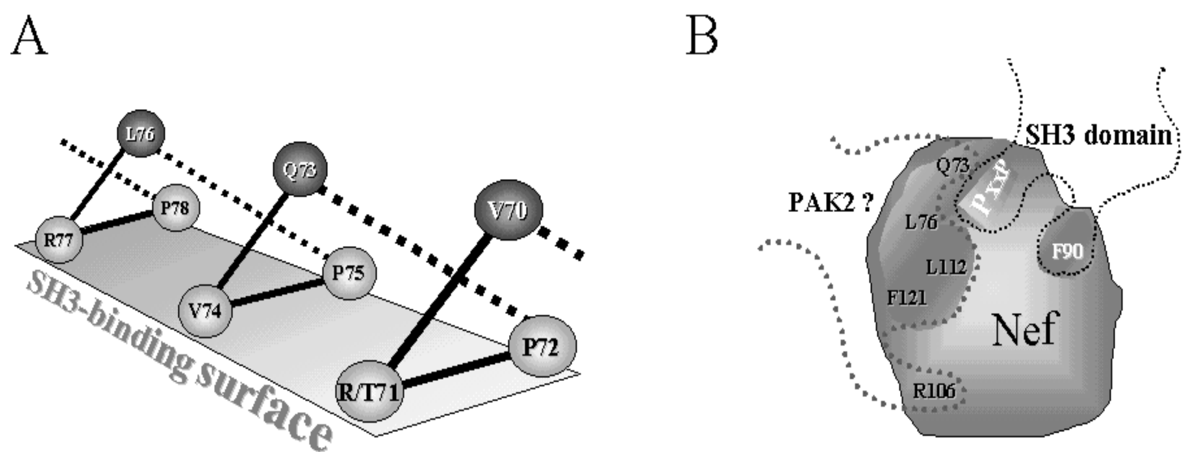

Figure 6. PxxP-mediated interactions of Nef with SH3 domains.

A) The PxxP-motif of NL4-3 Nef. Due to helical structure of the polyproline type II (PPII)-helix, Q73- and L76-residues (dark spheres) point away from the SH3-contacting surface (light spheres)
B) Additional strength and specificity for the PxxP-mediated binding of Nef to an SH3 domain is provided by a hydrophobic pocket that is in part formed by F90 residue (white fonts). Similarly, the capacity of Nef to coprecipitate PAK2 depends on the surfaces essential for SH3-binding (white fonts). This implies that Nef/NAK-interaction depends on an additional SH3-containing component. In addition, other residues, not involved in SH3-binding, are required for Nef/NAK-interaction (black fonts). These residues could directly participate in binding to PAK2.

Table 2. Summary of the Nef-mutant proteins used in study I

Category 1: Wild-type Nef variants	Nef-T71, Nef-R71
Category 2: Mutations in SH3-contacting residues within the PxxP-motif	Nef-AxxP {P72A}, Nef-PxxA {P75A}, Nef-AxxA {P72A;P75A}, Nef-V74D, Nef-R77E, Nef-VDRE {V74D;R77E}
Category 3: Changes in non-SH3-contacting residues within the PxxP-motif	Nef-P69A, Nef-QPLA {Q73P;L76A}
Category 4: Mutations in SH3-contacting residues outside the PxxP-motif	Nef-F90R
Category 5: Mutations in non-SH3-contacting residues outside the PxxP-motif	Nef-R106A, Nef-L112R, Nef-F121R

The effects of mutations on the SH3-binding capacity of Nef (I)

We studied the effects of these mutations on the prototypic PxxP/SH3-mediated binding between Nef and the SH3 domain of Hck by measuring the surface-plasmon resonance (SPR) with biacore biosensor apparatus as described in materials and methods. In agreement with earlier reports (Lee *et al.* 1995), the wild-type Nef alleles bound to the Hck-SH3 domain with high affinities. As expected, the Nef mutants in category 2 with changes in SH3-contacting residues had dramatically reduced SH3-binding capacity. Similarly, mutating the critical phenylalanine (Nef-F90R) contacting the RT-loop of the Hck-SH3 domain (category 4) reduced the affinity 10-fold as compared to the wild-type Nef-R71. The Nef mutants with changes in residues not expected to participate in SH3-binding (categories 3 and 5), on the contrary, bound to Hck-SH3 equally well as wild-type Nef variants.

The effects of mutations on the capacity of Nef to coprecipitate PAK2 (I)

To correlate the data representing the SH3-binding capacity of different Nef proteins to their ability to coprecipitate PAK2, we transiently expressed the same Nef mutant proteins in 293T cells followed by Nef-immunoprecipitation and an *in vitro* kinase assay. We found that only the wild-type Nef proteins (Nef-R71 and Nef-T71) efficiently coprecipitated PAK2. All of the Nef mutant proteins, which were negative in SH3-binding, failed to associate with PAK2 indicating

that an SH3-binding function of Nef is required. However, Nef proteins with mutations in residues that were not involved in SH3-binding (categories 3 and 5) also failed to coprecipitate PAK2. A similar finding has recently been reported by others (Craig *et al.* 1999).

We confirmed that an intact diarginine motif (category 5) in the core region of Nef is critical for association with PAK2 (Sawai *et al.* 1995). Nef-QPLA (category 3) was capable of binding to Hck-SH3 but did not associate with PAK2. By studying the available Nef/SH3-cocrystal and Nef-NMR data we found that Q73 and L76 together with several other residues (including the L112 and F121, category 5) form a hydrophobic surface that is exposed even when Nef is bound to an SH3 domain. It is possible that this hydrophobic domain participates in binding to PAK2 (Figure 6B). However, recent reports implicate the residues involved in the formation of this hydrophobic surface, as well as R106, in oligomerization of Nef (Arold *et al.* 2000, Liu *et al.* 2000). In such a scenario the effects of these mutations would be less direct as they disrupt the oligomeric structures of Nef that are thougth to be important for many of its cellular functions.

Another interesting residue is P69 (category 3) since we and others have shown it to be important for PAK2 interaction (**I**, Wiskerchen and Cheng-Mayer 1996). Despite being the first of four prolines in the tetraproline repeat of Nef, P69 does not contribute to SH3-binding. It is likely that the role of P69 is to correctly position the N-terminal flexible arm of Nef. Whether the goal is to prevent the N-terminus from interfering with the above-mentioned hydrophobic patch of Nef or to allow contribution of some N-terminal residues to these interactions is not known. An incorrectly bent N-terminus could also affect the membrane association of Nef. However, Nef-P69A retains its capacity to downregulate CD4 and is therefore most likely membrane-bound (Wiskerchen and Cheng-Mayer 1996, our unpublished data).

The functional motifs of PAK2 required for association with Nef (III)

As mentioned earlier, none of the PAK species have SH3 domains but they do have several other sequence motifs shown to mediate interactions with cellular proteins. The PxxP-motif in the amino terminus of PAK binds to Nck (Bagrodia *et al.* 1995b, Lu *et al.* 1997, Zhao *et al.* 2000), and the PIX-binding motif mediates interaction with β-PIX/Cool-1 (Bagrodia *et al.* 1998, Manser *et al.* 1998). Rac1 and Cdc42, bind to the CRIB-motif in the autoregulatory region of PAK (Burbelo *et al.* 1995, Thompson *et al.* 1998, Lei *et al.* 2000, Morreale *et al.* 2000). In addition, PAK2 is a substrate for DEVD-sensitive caspases (Rudel and Bokoch 1997). Assuming that Nef and PAK2 interact with each other in a multimolecular complex, exploring the roles of these functional domains would help to define other proteins necessary for stabilizing Nef/PAK2-interaction.

By generating chimeric proteins containing parts from both PAK1 and PAK2, we showed that the carboxyterminal part of the autoregulatory region of PAK2 mediated association with Nef. In agreement with the differential capacity of PAK1 and PAK2 to associate with Nef, this fragment is also one of the most dissimilar regions between the PAK isoforms. However, it is possible that some of the functional motifs outside this region may have additional roles in Nef/PAK2 interaction. These functional domains could be important for recruitment of additional components or for modification of the activity and/or the conformation of PAK2 itself. A guanine exchange factor VAV has been reported to directly bind to Nef both in cells and *in vitro* (Fackler *et al.* 1999). Fackler *et al.* proposed a model where Nef, NAK, Cdc42/Rac1 and VAV together form a signaling complex. This complex activated JNK/p38-cascade and cytoskeletal rearrangements, both of which may contribute to increased viral replication (Fackler *et al.* 1999). Another candidate is an adaptor protein Nck whose second SH3 domain (of three) has been shown to be able to bind to NAK (**I**). Additionally, PAK-interacting exchange factor (β-PIX) contains an SH3 domain (Manser *et al.* 1998).

We found that the PxxP-mutant, the caspase-mutant and the PIX-mutant efficiently substituted for endogenous NAK. Thus, the interaction of PAK2 with Nef does not depend on its capacity to interact with Nck, β-PIX or caspases. Consequently, the roles of cellular proteins that interact with PAK2 through these factors can also be considered dispensable for association with Nef. Moreover, our attempts to detect interaction between Nef and Nck have been unsuccessful (unpublished data). In contrast, the CRIB-motif in the aminoterminal part of the autoregulatory region of PAK2 was found to be essential for association with Nef. Interestingly, we also showed that NAK represents a highly active yet a small subpopulation of PAK2. Such a form of PAK2 can be induced by Cdc42-mediated activation. Thus, the CRIB-mediated activation of PAK2 is required to make PAK2 a NAK. The requirement for a functional CRIB-motif is interesting in light of the data showing functional interaction between VAV and Nef (Fackler *et al.* 1999). However, in our preliminary experiments we have not been able to confirm these findings (unpublished data).

7.1.2. The Nef/PAK2-interaction and viral replication

The role of PAK2 in the pathogenesis of AIDS is still unclear. Studies in SIV-infected monkeys have yielded contradictory results (Sawai *et al.* 1996, Lang *et al.* 1997, Khan *et al.* 1998). The involvement of PAK2 in apoptotic signaling (Rudel and Bokoch 1997) provides a novel putative mechanism by which Nef could protect the host cell from apoptosis (Xu *et al.* 1997, Xu *et al.* 1999). Apoptosis in Jurkat T cells induced a Cdc42/Rac1-independent activation of PAK2 by caspase-mediated cleavage (Rudel and Bokoch 1997). Moreover, cells

overexpressing a dominant-negative mutant of PAK2 did not form apoptotic bodies upon Fas-triggered apoptotic signal (Rudel and Bokoch 1997). Therefore, in order to protect the host cell from Fas-mediated apoptosis Nef would have to inhibit PAK2 activation. On the other hand, only highly active PAK associates with Nef (**III**). This does not necessarily mean that Nef would activate PAK2-mediated signaling. Although such reports have been published (Lu *et al.* 1996, Sawai *et al.* 1996, Brown *et al.* 1999, Arora *et al.* 2000, Fackler *et al.* 2000), it is possible that Nef inhibits PAK2 signaling by sequestering all of the available highly active PAK2. In this context, it should be noted that, regardless of the stimulus, only a small fraction of the total cellular pools of PAK2 are modified to this highly active form (**III**). PAKs have also been implicated in regulation of the cytoskeletal organization (Bagrodia and Cerione 1999). Consequently, Nef-mediated modulation of PAK2 activity might facilitate the assembly and/or budding of the progeny virions.

7.2. Nef and T cell signaling (IV, V, VI)

HIV-1 Nef synergizes with the Ras/MAPK-pathway to activate NFAT (IV)

To study the effects of ectopic Nef expression on T cell signaling we set up a transient transfection assay in Jurkat T cell line and used the nuclear factor of activated T cells/antigen receptor response element of the interleukin-2 gene (NFAT/ARRE-2)-luciferase reporter system as a read-out for T cell activation (see Figure 4, page 19). Because stable Nef expression is generally toxic in T cell lines (e.g. Baur *et al.* 1994 and our unpublished data) we reasoned that by transient expression system we could more reliably analyze the effect of Nef since stable Nef-expressing cell lines might show altered growth properties. Moreover, such a transient expression profile mimics probably better the situation during the acute phase of productive HIV infection.

We found that Nef did not significantly modulate TCR- or PMA+ionophore-triggered activation of NFAT-driven transcription. The basal NFAT activity was also not affected by Nef expression. In contrast, when Nef-expressing cells were stimulated only with PMA, an up to 100-fold increase in NFAT-driven transcription was observed. These results suggested that Nef activated the Ca^{2+}/calcineurin pathway and synergized with the Ras/MAPK-cascade to efficiently activate NFAT-dependent transcription. Furthermore, we showed that Nef induced nuclear targeting of co-expressed GFP-tagged NFATc1 (**VI**).

The NFAT-activating effect requires relatively high expression levels of Nef. High expression levels were obtained in T cells by using an elongation factor 1α-promoter-driven Nef-expression vector. This could be one of the reasons why others employing less efficient expression systems (such as cytomegalovirus (CMV)- promoter-based vectors) have not seen such an effect by Nef. In this

respect, it will be important to determine the cellular levels of Nef-expression during the acute phase of productive HIV infection. Estimations based on data from chronically infected cell cultures suggest that the levels are relatively high (Wang *et al.* 2000) but they might still underestimate the levels existing during the acute phase. Another reason for different results may be that whereas several earlier studies have used stable Nef-expressing cell clones, we have used transient transfection-based expression of Nef. The Nef-expressing cell clones must have adapted to growth in the presence Nef. However, the cytotoxic properties of Nef may be related to its effects on cellular signaling.

Coexpression of Nef and a constitutively active form of PCKθ efficiently activate NFAT (V)

Phorbol ester treatment activates several cellular signaling pathways, especially those pathways controlled by members of the protein kinase C (PKC) family (Ron and Kazanietz 1999, Kazanietz 2000). To date, 11 PKC isozymes are known and most of them are expressed in T cells (Baier *et al.* 1993, Hug and Sarre 1993). We examined whether the effects of overexpression of constitutively active forms of different PKC isoforms can substitute for the Ras/MAPK-inducing effect of PMA. We found that although coexpression of Nef and PKCθ-A148E strongly activated NFAT in Jurkat cells, the cooperative capacity of PKCδ-A147E was less than 20 percent of that of PKCθ-A148E and that the constitutively active form of PKCα was virtually unable to activate NFAT. The differential abilities of these PKC isoforms to cooperate with Nef were not due to their different intrinsic activities because all three activated serum response element (SRE)-driven transcription roughly to the similar degree when expressed in 293T fibroblasts.

It is interesting to note that Smith *et al.* have described physical and functional interaction between Nef and PKCθ (but not other PKC isoforms) (Smith *et al.* 1996). However, they found that, in contrast to synergistic effects seen in our studies, Nef downregulated the steady-state expression levels of PKCθ and interfered with its activation (Smith *et al.* 1996). On the other hand, their observations could also be interpreted in the opposite way. Activation of PKCθ by mitogens or co-expression of PKCθ with Nef, both seem to result in translocation of PKCθ into the particulate fraction (Smith *et al.* 1996). Therefore it is possible, that in Nef-expressing cells PKCθ in the particulate fraction represents an active pool. In this case the effects of Nef on PKCθ signaling could be positive.

Despite the plurality (and similarity between certain isoforms) of PKC isoforms expressed in T cells, as well as in many other cell types, they seem to have relatively specific roles. A large body of data suggests for a crucial role of PKCθ in T cell activation. Firstly, the expression pattern of PKCθ is restricted mainly to

T cells (Meller *et al.* 1998). Secondly, PKCθ is the only PKC isozyme that is recruited to the supramolecular activation complexes (SMACs or immunological synapses) followed by TCR-ligation (Monks *et al.* 1997, Monks *et al.* 1998). Finally, Werlen *et al.* reported that calcineurin preferentially synergizes with the PKCθ isozyme to activate JNK and IL-2 promoter (Werlen *et al.* 1998) and Villalba *et al.* provided evidence of functional interaction between PKCθ and VAV that is crucial for activation of AP-1 and NFAT in T cells (Villalba *et al.* 2000). Furthermore, we saw similar hierarchical capacity of PKC isoforms ($\alpha < \delta < \theta$) to synergize in the activation of NFAT when calcium ionophore was used to trigger calcium signaling (unpublished data). Thus it seems likely that the superior cooperative capacity of PKCθ with Nef reflects its better compatibility with signal transduction pathways involved in TCR-signaling rather than a specific physical and/or functional interplay with Nef.

Nevertheless, activation of PKCθ is sufficient to generate a signal that can cooperate with Nef to upregulate expression of the target genes of NFAT, suggesting that in Nef-expressing T lymphocytes any signal or situation leading to induction of PKCθ activity may be able to trigger a complete T cell activation program. Interestingly, the positive effect of Nef on viral replication in primary cell cultures is most pronounced in suboptimally stimulated cultures (Miller *et al.* 1994, Spina *et al.* 1994). A possible *in vitro* model for such abnormal T cell activation is provided by Alexander *et al.* who found that in Herpesvirus saimiri transformed T cell line, Nef (+) viruses were able to replicate in the absence of exogenously added IL-2 (Alexander *et al.* 1997). This was due to Nef-induced autocrine production of IL-2 by the infected cells (Alexander *et al.* 1997). The T cell immortalizing capacity of Herpesvirus saimiri (HSV) has been mapped to a single oncogene STP-C488 that interacts with and activates cellular Ras (Jung and Desrosiers 1995). Furthermore, Guo *et al.* demonstrated that recombinant HSV with a constitutively active Ras gene substituted for STP-C488 was capable of immortalizing primary T lymphocytes (Guo *et al.* 1998). Thus in SIV-infected, HSV-immortalized primary T cells Nef and STP-C488 could trigger the Ca^{2+}/calcineurin- and Ras/MAPK-pathways, respectively, leading to efficient induction of NFAT-driven transcription and subsequent upregulation of IL-2, allowing replication of Nef-containing viruses in these cultures without antigenic or other related (αCD3, lectin) stimuli.

Nef-mediated activation of Ca^{2+}/calcineurin signaling is independent of TCR-proximal signaling events (IV)

To further characterize the nature of this positive effect by Nef on T cell activation, we co-transfected dominant-negative (DN) forms of signaling molecules known to act at distinct steps in signaling cascades triggered by TCR. The effects of these proteins on Nef+PMA-mediated NFAT activation were compared in parallel with TCR-triggered (αCD3-treatment) activation of NFAT.

We found that the effect of Nef on calcium signaling was independent of two proteins, Lck and PAK1, both involved in early events of TCR-mediated signaling (Mustelin 1994, Yablonski *et al.* 1998). On the other hand, calcineurin function and Ca^{2+}-influx were required. Although our data from Lck-deficient Jurkat cells suggest no role for Lck, Fyn, another Src family member, has been associated with regulation of intracellular calcium homeostasis (Jayaraman *et al.* 1996). However, by using an inhibitor of the Src-family tyrosine kinases, PP1, we found that Fyn, like Lck, does not seem to be required for Nef-mediated activation of Ca^{2+}/calcineurin signaling. Furthermore, overexpression of a lipase-inactive PLCγ1 did not affect Nef+PMA-mediated activation of NFAT (**VI**). Thus, activation of the Ca^{2+}/calcineurin pathway by Nef represents a novel function that is distinct from the previously reported positive effects of Nef on TCR-mediated signaling (Schrager and Marsh 1999, Wang *et al.* 2000).

The NFAT-activating effect of Nef requires inositol trisphosphate receptor (IP_3R) function (VI)

Because we had shown that the NFAT-activating effect of Nef was independent of several TCR-proximal events but required elevated calcium levels and subsequent activation of calcineurin function, our mechanistic studies were focused on the capacitative calcium entry (CCE) system (Guse 1998, see page 20 and figure 7). We found that blocking IP_3R function with 2-aminoethoxydiphenyl borate (2-APB), a specific inhibitor of IP_3Rs (Maruyama *et al.* 1997), completely abolished activation of NFAT by Nef+PMA. Similarly, Nef did not synergize with PMA in a Jurkat cell clone stably expressing an antisense-RNA for IP_3R1. Moreover, Nef was found to coprecipitate with IP_3R1. Therefore it is possible that by activating IP_3R function, Nef causes depletion of the intracellular Ca^{2+}-stores and consequently activation of Ca^{2+}-influx. Surprisingly, we found that the IP_3-sensitive stores were unmodulated in Nef-expressing cells. These findings could be best explained by a direct IP_3R/SOC coupling model (Figure 7). In such a scenario Nef would facilitate the physical contact between these channels by either directly bridging them or possibly by modulating the conformation of the IP_3R. The latter alternative assumes that the depletion of intracellular Ca^{2+}-stores results in a specific conformation of IP_3R that mediates activation of CCE. Nef may induce such a conformation independently of the intracellular Ca^{2+}-store content. However, another possibility is that the IP_3-sensitive stores responsible for activation of CCE may represent only a minor fraction of the total Ca^{2+}-stores (Huang and Putney 1998) and therefore it may be difficult to detect depletion of these specialized Ca^{2+}-stores.

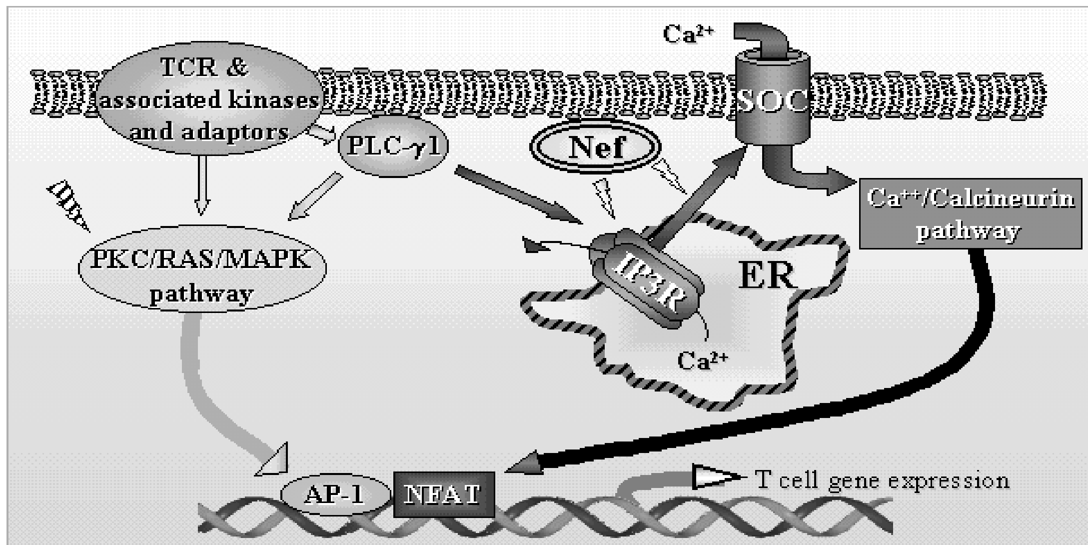

Figure 7. A model for Nef-mediated activation of NFAT

Nef may facilitate the IP_3R-dependent but Ca^{2+}-release-independent activation of CCE by serving as a bridging molecule between IP_3R and SOC. Alternatively, Nef may induce a specific conformation of IP_3R that is required for activation of SOCs. Co-incidental activation of the PKC/Ras/MAPK-pathway (striped flash) would therefore lead to efficient activation of NFAT-driven gene expression.

Although Nef clearly induced the transcriptional activity of NFAT (**IV**), the Nef-mediated elevation in the intracellular Ca^{2+}-levels ($[Ca^{2+}]_i$) is likely to be small because we did not detect such an elevation by aequorin-based Ca^{2+}-measurement system or by FACS analysis of cells loaded with fluorescent Ca^{2+} indicator dyes (**VI,** and unpublished data). However, although sustained elevated Ca^{2+}-levels have been implicated in the activation of NFAT (Timmerman *et al.* 1996, Dolmetsch *et al.* 1997), additional regulatory mechanisms have been reported (Dolmetsch *et al.* 1998, Li *et al.* 1998b). These studies demonstrated that if Ca^{2+} signals were triggered as a series of oscillating Ca^{2+} spikes with an appropriate frequency, remarkably low average levels of intracellular calcium ($[Ca^{2+}]_i$ less than 200nM) elicited efficient activation of NFAT. By inducing formation of such Ca^{2+}-spikes Nef could activate NFAT without significant increase in the averaged steady-state $[Ca^{2+}]_i$. Importantly, these data indicate that the transcriptional assay for NFAT activity serves as a sensitive measure of intracellular calcium metabolism given that the Ras/MAPK-pathway activating signal is provided (e.g. by PMA).

Activation of Ca^{2+}/calcineurin signaling is a conserved function of Nef (V)

To study whether the activation of NFAT is a conserved function of Nef, we transfected five different HIV-1 Nef variants (NL4-3, NL4-3(R71), BH10, SF2 and HAN-2) and an SIV Nef (mac239) into Jurkat cells and stimulated these cells with PMA. All the different Nef proteins were able to synergistically activate NFAT, indicating that this function of Nef is conserved in different primate lentiviruses such as HIV and SIV.

The SH3-binding function and membrane targeting of Nef are required for Nef-mediated activation of NFAT (V)

To study the possible correlation of Nef-mediated activation of NFAT with the other previously described functions of Nef, we characterized the regions of Nef involved in mediating the NFAT-activating function. We analyzed several Nef mutant proteins (chosen based on their known phenotype) for their capacity to synergize with PMA to activate NFAT. Our studies indicated that membrane association and SH3-binding function of Nef were required. Additionally, several other residues that were not involved in SH3-binding but were found to be important for PAK2 association (**I**) were required for activation of NFAT by Nef. In contrast, Nef elements implicated in CD4 downregulation by connecting Nef to the endocytotic machinery were found to be dispensable for the ability of Nef to regulate NFAT. Similarly, the putative PKC-phosphorylation sites in the N-terminus of Nef were not required for the Nef-mediated activation of NFAT.

7.2.1. Nef-mediated activation of NFAT in T cells – implications on viral replication

Our finding that the ability of Nef to activate the Ca^{2+}/calcineurin pathway (leading to the activation of NFAT) is a conserved function of different Nef variants suggests that it may have an important role in contributing to the pathogenic properties of Nef. Interestingly, a mere overexpression of NFATc1 in primary CD4-positive T cells by means of retroviral gene transfer renders these cells susceptible for HIV replication (Kinoshita *et al.* 1998). Thus overexpression of NFATc1 that per se leads to its partial nuclear translocation in Jurkat T cells (our unpublished observation), overcomes a block that normally inhibits HIV replication at reverse transcription phase in the absence of T cell activating

stimuli (Kinoshita *et al.* 1998). However, an obvious dilemma in such a scenario is that it seems likely that the integration of the viral genome has to precede efficient expression of Nef (Sakai *et al.* 1993, Engelman *et al.* 1995, Cara *et al.* 1996). Moreover, Aiken *et al.* have reported that ectopic Nef expression in the target cells cannot rescue the Nef-phenotype when these cells are infected with Nef-deleted viruses (Aiken and Trono 1995). Although a few copies of Nef molecules are incorporated into virions, the majority of them are cleaved by viral protease into apparently inactive forms (Pandori *et al.* 1996, Welker *et al.* 1996). In light of these data, it seems unlikely that Nef would modulate the cellular environment of the host cell prior to integration.

Nevertheless, the beneficial effects of Nef-mediated NFAT activation on HIV-replication could also be attributed to the post-integration step. Kinoshita *et al.* reported that NFATc binds to an uncommon NFAT consensus site on HIV-long terminal repeat (LTR) and positively regulates its transcriptional activity (Kinoshita *et al.* 1997). Apart from the possible direct effects on HIV gene expression, NFAT participates in the regulation of several cellular genes such as IL-2, IL-4, tumour necrosis factor (TNF)-α and FasL, which are centrally involved in T cell activation, effector functions, and apoptosis (Shaw *et al.* 1988, Goldfeld *et al.* 1993, Szabo *et al.* 1993, Latinis *et al.* 1997). The modulation of these cellular activities, all of which are intimately involved in the pathogenesis of HIV infection, could have indirect effects on the replication of the virus.

The mutagenesis data showed that the regions of Nef required for the NFAT activation correlated with those critical for the enhancement of viral replication and virion infectivity by Nef (**V**). The requirement for an intact PxxP-motif as well as for residues implicated in association with PAK2 seems to be a reoccurring feature of several Nef functions (Renkema and Saksela 2000). Facing the steadily increasing number of Nef functions dependent on the SH3-binding capacity, it is tempting to speculate that the PxxP-motif of Nef may serve as a general regulator of Nef function rather than a docking site for various Nef effector molecules. This regulation could be exerted, for example, by recruiting Nef into specific intracellular location or by mediating interaction with a single partner that modulates and activates Nef. Similarly, residues participating in the formation of the hydrophobic patch (along with R106) may be required merely for the oligomerization of Nef that is thought to be crucial for functional activation of Nef (Liu *et al.* 2000).

Other scientists have reported positive effects of Nef on T cell activation (Baur *et al.* 1994, Du *et al.* 1995, Luo and Peterlin 1997, Schrager and Marsh 1999, Wang *et al.* 2000). There are, however, some fundamental differences when comparing their results with ours (**IV**, **V, VI**). The positive effect of Nef, found in the above-mentioned studies is exerted on TCR-complex whereas our data strongly suggests that the NFAT-activating function of Nef is independent of TCR-proximal events. Some differences in experimental settings are noteworthy. Baur *et al.* demonstrated that Nef could either activate or inhibit TCR-signaling depending on its apparent intracellular location (Baur *et al.* 1994). The authors used a chimeric CD8-Nef protein (the extracellular and transmembrane domains of CD8 fused to the N-terminus of Nef) to force the surface expression of Nef. This complicates the interpretation of these results as it is not known how well the effects of such chimera are reflected by native Nef variants. Similarly, the activating effect of the SIVpbj14-Nef is mediated by an immunoreceptor tyrosine-based activation motif (ITAM) that is not found in other Nef variants (Du *et al.* 1995, Luo and Peterlin 1997).

Nef-mediated lowered threshold for the triggering of the TCR/CD28-mediated signaling was reported by two studies (Schrager and Marsh 1999, Wang *et al.* 2000). Nef did not synergize with phorbol esters to activate T cell signaling nor did it elicit any signal by itself and it was suggested that Nef might augment TCR-response by physically bridging some of the key components of the TCR-complex (Schrager and Marsh 1999, Wang *et al.* 2000). To this end, it was shown that Nef associates with rafts (Wang *et al.* 2000) and that the enhanced production of IL-2 by Nef-expressing T cells was due to an increased number of cells responding to antigenic stimulus rather than higher levels of IL-2 produced per cell (Schrager and Marsh 1999). We did not observe synergistic effects of Nef with TCR-triggered stimulus either with suboptimal concentrations of TCR-activating antibodies or with different amounts of transfected Nef. These two studies were mainly based on data from cells exposed to long-term Nef-expression. We instead have used a transient transfection approach and thus further studies are needed to reveal the reasons behind these different observations.

Foti *et al.* showed that the intracellular Ca^{2+} store content was increased in Nef-expressing myelomonocytic cells (Foti *et al.* 1999). This effect was found only in

differentiated myelomonocytic HL60 cells and correlated with the expression levels of Hck, as well as the capacity of Nef to bind to this Src-family kinase. The authors suggested that Hck, activated by Nef, interacts with IP_3R thereby modulating the cellular calcium metabolism (Foti *et al.* 1999). The intracellular Ca^{2+} store content in undifferentiated HL60 cells or in CEM T lymphocytes was unchanged by Nef expression. On the contrary, we did not observe differences in the Ca^{2+} store content in transiently Nef-transfected Jurkat T cells (**VI**). Moreover, several lines of evidence argued against a role for Src-family tyrosine kinases as mediators of the NFAT-activating function of Nef (**IV**).

The essential role of Nef in the pathogenesis of AIDS makes it an attractive target for anti-HIV drug design. The development of drugs specifically interfering with Nef requires detailed information about the mechanisms by which Nef exerts its effects. The identification of NAK as PAK2 will certainly facilitate the characterization of the Nef/NAK-interaction and its importance for viral replication. The novel TCR-independent effect of Nef on T cell activation described in this study may provide new approaches to inhibit Nef function and restrict HIV replication.

8. SUMMARY

Nef, an accessory protein of HIV and SIV, has been under active investigation ever since its central role in the pathogenesis of AIDS was described. Regardless of its small size, Nef seems to affect a variety of cellular processes by interacting, directly or indirectly, with a number of host cell proteins. Nef does not possess intrinsic enzymatic activity but rather seems to function as an adaptor protein or as a molecular switch that via physical interactions modulates the activity and/or localization of cellular proteins. Such an exploitation of host cell activities has made it difficult to identify the critical Nef interactions because of the multimolecular nature of most signaling events and extensive redundancy of the cellular signaling networks.

In the present study we have shown that the Nef-associated cellular serine/threonine kinase (NAK) is p21-activated kinase 2 (PAK2). Furthermore, we have characterized the structural and functional features required for their interaction. Our data showed that the SH3-binding domain as well as another site of Nef was required for this interaction. Since PAK2 does not contain SH3 domains, we concluded that an additional SH3-containing component is likely to participate in the formation of Nef/PAK2-complex. The identification of NAK as PAK2 facilitates the studies aiming at understanding the mechanistic role of NAK in Nef-mediated effects on viral pathogenicity. We also showed that an intact CRIB-motif in PAK2, important for PAK activation, was required for interaction with Nef. Interestingly, we demonstrated that Nef specifically associated with a highly active small subpopulation of PAK2.

The other main area of investigation was the effect of Nef on T cell activation. We found a novel function of Nef that was conserved among different Nef variants, thus suggesting an important role for this function *in vivo*. Nef activated the Ca^{2+}/calcineurin signaling in a manner that was independent of T cell receptor proximal events but required inositol trisphosphate receptor function. Consequently, Nef-mediated activation of calcium signaling was strongly synergistic with activation of the Ras/MAPK-pathway in induction of NFAT-driven transcription. Additionally, protein kinase C-θ (PKCθ) was implicated as a possible physiological cofactor for Nef in NFAT activation. Furthermore, by employing a mutagenesis approach, we found that an intact SH3-binding domain,

membrane targeting and residues implicated in PAK2 binding and oligomerization of Nef, were required for this function. The finding that Nef participates in NFAT activation could be highly relevant for the capacity of Nef to enhance viral replication. Clearly, development of biological models to study this feature of Nef in a viral context is important. In addition to its impact on HIV studies, our observation that Nef activates Ca^{2+}-influx without apparent depletion of the intracellular Ca^{2+}-stores provides new insights into studies addressing the role of IP_3R in mediating capacitative calcium entry (CCE).

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11. ORIGINAL COMMUNICATIONS