



KATI PULKKINEN

HIV-1 Nef Protein and  
the Nef-associating Kinase PAK2  
in Cell Signaling



ACADEMIC DISSERTATION

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for public discussion in the Jarmo Visakorpi Auditorium,  
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ACADEMIC DISSERTATION

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## **ABSTRACT**

Nef is an accessory protein encoded by the human and simian immunodeficiency viruses (HIV/SIV) and is a major factor in promoting viral pathogenesis. Several cellular functions of Nef, including downregulation of the surface molecules CD4 and MHC I, increase in viral infectivity, and Nef-mediated effects on intracellular signaling favor survival and propagation of the virus. Nef does not possess any intrinsic enzymatic activity; instead, it functions by interacting with a number of endogenous proteins within the host cell.

One of the well-characterized Nef-associating protein partners and a central molecule in this study is p21-associating kinase 2 (PAK2). Previously, association of the HIV-1 Nef with PAK2 has been proposed to play a role in T cell activation, viral replication, apoptosis and progression of HIV infection to AIDS. Despite intense research, there is no consensus on the relevance of Nef-PAK2 association and its function in virus pathogenesis. Furthermore, selective association of Nef with a minute but highly active subpopulation of total cellular PAK2 has raised questions on the molecular requirements of Nef-PAK2 association. This work elucidated the interplay of Nef and PAK2 in cellular signaling by studying the mechanism of PAK2 activation and its role in Nef association. To clarify the role of additional proteins during Nef-PAK2 complex formation, the involvement of p21-GTPases and guanine nucleotide exchange factor (GEF) proteins was studied. Finally, the functional role of charge distribution within the putative Nef interaction surface was characterized.

Here, we describe a new signaling pathway that potentiates PAK2 activation by converging signal transduction via Src tyrosine kinases with p21-GTPase-induced PAK activation. The p21-GTPase-induced conformational opening of PAK2 was associated with the ability of PAK2 to interact with Nef, and their association was stabilized by reduced kinase activity. The Nef-PAK2 (NAK) complex was specifically localized to detergent-resistant membranes (DRM), and Vav1 was identified as a GEF component of Nef-PAK2 complex, recruited to the DRMs by Nef. The reciprocal charges present at the putative dimer interface (PDI) of Nef were indispensable for Nef-dependent increase in viral infectivity. In addition, several other Nef activities, including NAK association, membrane localization, and activation of Hck were coordinated by charged residues at the Nef PDI.

In conclusion, our results provide new insight on the cellular signaling upstream of Nef-PAK2 association, as well as on the molecular domains and binding partners that are important for the formation of the Nef-PAK2 complex. In addition, our data elucidate how Nef functions are related to charge distribution at the PDI surface. Detailed knowledge on the molecular mechanisms involved in Nef-PAK2-mediated

signaling may provide clues for potential target sites that can be used to develop HIV inhibitory drugs.

## LIST OF ORIGINAL COMMUNICATIONS

The thesis is based on following original scientific communications, which are referred to in the text by their Roman numerals I-IV.

- I Renkema GH, **Pulkkinen K** and Saksela K (2002). Cdc42/Rac1-mediated activation primes PAK2 for superactivation by tyrosine phosphorylation. *Mol Cell Biol* 22: 6719-6725.
- II **Pulkkinen K**, Renkema GH, Kirchhoff F and Saksela K (2004). Nef associates with p21-activated kinase 2 in a p21-GTPase-dependent dynamic activation complex within lipid rafts. *J Virol* 78: 12773-12780.
- III Rauch S, **Pulkkinen K**, Saksela K and Fackler OT (2008). Human immunodeficiency virus type 1 Nef recruits the guanine nucleotide exchange factor Vav1 via an unexpected interface into plasma membrane microdomains for association with p21-activated kinase 2 activity. *J Virol* 82: 2918-2929.
- IV **Pulkkinen K**, Tibroni N, Fackler O, Saksela K (2009). Functional role of HIV-1 Nef self-association elucidated via reciprocal charge substitutions at the putative Nef dimer interface. *Manuscript submitted*.



## ABBREVIATIONS

Aa	amino acid
ADF	actin depolymerizing factor
AID	autoinhibitory domain
AIDS	acquired immunodeficiency syndrome
AP	adaptor protein
APC	antigen-presenting cell
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ARF	ADP ribosylation factor
bp	base pair
$\beta$ -COP	$\beta$ subunit of COPI coatomers
CA	capsid protein
CAT	COOL-associated, tyrosine phosphorylated
CD	cluster of differentiation
Cdc42	cell division cycle 42
COOL	cloned out of library
CRIB	Cdc42/Rac interactive binding domain
CTL	cytotoxic T lymphocyte
CTx	cholera toxin
DNA	deoxyribonucleic acid
DRM	detergent-resistant membrane
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
Env	envelope protein
ER	endoplasmic reticulum
FCS	fetal calf serum
G-CSF	granulocyte colony-stimulating factor
GEF	guanine nucleotide exchange factor
GIT	G protein-coupled receptor kinase-interactor
Gp	glycoprotein
GST	glutathione <i>S</i> -transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HAART	highly-active anti-retroviral therapy
Hck	hematopoietic cell kinase
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HSC	hematopoietic stem cell
hTE	human thioesterase
HTLV	human T cell leukemia virus
IN	integrase
IP3R	inositol trisphosphate receptor
IS	immunological synapse
IVKA	<i>in vitro</i> kinase assay
JNK	c-Jun N-terminal kinase
Kb	kilobase
kD	kiloDalton
LNTP	long-terminal non-progressor
LTR	long terminal repeat
MA	matrix protein

MAP/ERK	mitogen-activated protein/extracellular signal-regulated protein kinase
MHC I	major histocompatibility class I
MLCK	myosin light chain kinase
mRNA	messenger-RNA
NAK	Nef-associated kinase
NC	nucleocapsid protein
Nef	negative factor
NF- $\kappa$ B	nuclear factor $\kappa$ B
NFAT	nuclear factor of activated T cells
ORF	open reading frame
PACS	phosphofurin acidic cluster sorting protein
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PBD	p21-GTPase binding domain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDI	putative dimer interface
PDK	pyruvate dehydrogenase kinase
PI3K	phosphoinositide-3-kinase
PIC	preintegration complex
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PIX	PAK-interacting exchange factor
PKC	protein kinase C
PKL	paxillin kinase linker
PMSF	phenylmethylsulfonyl fluoride
Pol	polymerase protein
POPX	partner of PIX
PR	protease
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]- pyrimidine
P-TEF-b	positive transcriptional elongation factor b
PTP-1B	protein tyrosine phosphatase 1B
PV	pervanadate
PVDF	polyvinylidene difluoride
Rev	regulator of viral protein expression
R-MLC	regulatory myosin light chain
RNA	ribonucleic acid
RRE	Rev-responsive element
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SFK	Src-family tyrosine kinase
SH3	Src homology 3
SIV	simian immunodeficiency virus
SMAC	supramolecular activation cluster
SP	slow progressor
Tat	transactivator protein
TcR	T cell receptor
TfR	transferrin receptor
TGN	<i>trans</i> -Golgi network
TNF	tumor necrosis factor
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
ZAP-70	zeta chain associated protein 70 kDa

## INTRODUCTION

Nef is an accessory protein of the human and simian immunodeficiency viruses (HIV/SIV). *In vivo*, Nef has an essential role in promoting viral replication and the subsequent evasion of host immune defense to develop AIDS. The critical property of Nef is its ability to associate with a number of endogenous proteins and by doing so, modulate cellular signaling cascades within the host cell to the benefit of the virus. The Nef functions that have been best characterized include downregulation of the T cell surface marker CD4 and major histocompatibility class I (MHC I) molecules, enhancement of viral replication kinetics, and intrinsic infectivity of HIV particles. In addition, Nef has various effects on T cell signaling.

One potentially important cellular interaction partner of Nef is Nef-associating serine/threonine kinase, identified as p21-activated kinase 2 (PAK2). The ability to associate with active PAK2 is a conserved property of Nef alleles derived from both HIV-1/-2 and SIV strains. However, Nef interacts with only a small subpopulation of the total cellular pool of active PAK2, suggesting that the differences in molecular properties, such as the phosphorylation status and conformation of PAK2, govern this selective association.

Nef associates with lipid rafts, which are specific membrane microdomains important for docking various proteins that are involved in signal transduction. In that view, it is not surprising that Nef association with PAK2 has been described to take place within a multiprotein complex, also referred to as 'signalosome' and several GEFs may be components of this complex. As an adaptor for many signals initiated by ligand binding and subsequent dimerization of receptor molecules, Nef has been also suggested to form oligomers in order to increase the intensity of downstream signaling.

The purpose of this work was to study the detailed molecular mechanism of PAK2 activation and the determinants of PAK2 and Nef association. In addition, we have characterized the intracellular localization of Nef-PAK2 complex and identified the GEF component of this complex. By studying the putative Nef dimerization interface, we have clarified the role of complementary charges within this interface in some of the most important Nef functions.

## REVIEW OF THE LITERATURE

### 1. Human and simian immunodeficiency viruses

Human immunodeficiency viruses 1 and 2 (HIV-1 and -2) and their primate counterpart, simian immunodeficiency virus (SIV) belong to the lentivirus genus within the *Retroviridae* family. The genus name, *Lentiviridae*, refers to the characteristically slow and progressive infection caused by these RNA viruses. The transmission of SIV from its original hosts, the African monkeys, to humans gave rise to different strains of HIV-1, which closely resemble the SIVcpz strain that infects chimpanzees (Gao *et al.*, 1999; Hahn *et al.*, 2000). The less pathogenic HIV-2 originates from a separate transmission of the SIVsm strain from sooty mangabeys to humans (Gao *et al.*, 1992; Marx *et al.*, 1991).

Lentiviruses became general knowledge in 1983, when researchers at the Pasteur Institute identified a novel T-lymphotropic retrovirus from a patient with lymphadenopathy (Barre-Sinoussi *et al.*, 1983). Soon, Robert Gallo's group at the NIH confirmed this finding, and the virus was associated with the acquired immunodeficiency syndrome, AIDS (Gallo *et al.*, 1983).

In 26 years, HIV infection has become a worldwide pandemic with an estimated 33 million people globally living with the virus ([www.unaids.org](http://www.unaids.org)). HIV-infected individuals develop severe defects in their immune system, suffer from various opportunistic infections and often develop cancer. Eventually, the immune dysfunction resulting from gradual depletion of CD4(+) T helper cells leads to disturbed function of cytotoxic T lymphocytes (CTL) and B cells. If untreated, the silent infection can become the terminal phase of the full-blown disease, AIDS, in approximately 10-12 years. Highly-active anti-retroviral therapy (HAART) has successfully treated infected patients by restricting virus replication. However, current drug regimens do not eradicate the virus from the body, thus making HIV treatment a life-long commitment. Furthermore, current drugs are economically inaccessible to the majority of patients living in developing countries, indicating the need for new approaches in drug development.

#### 1.1. HIV-1 genome

The 9.4-kB RNA genome of HIV consists of nine open reading frames (ORF) (Figure 1.). Three genes, *gag*, *pol*, and *env*, encode polyproteins that require further proteolysis into individual proteins. The HIV-1 proteins can be divided by their functions into three separate groups: structural (Gag, Env), regulatory (Pol, Rev, Tat), and accessory (Vif, Vpr, Vpu, Nef) proteins.

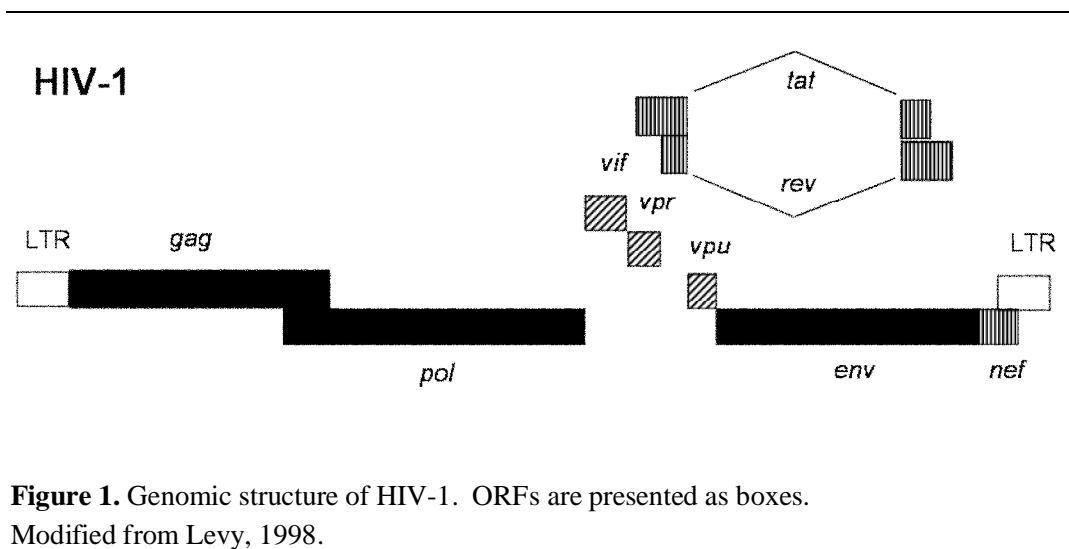
The *gag* gene encodes a polyprotein precursor, Pr55Gag, which is cleaved by the viral protease to yield the mature Gag proteins dubbed matrix (also known as MA/p17), capsid (CA/p24), nucleocapsid (NC/p7), and p6, as well as two small spacer peptides (p1 and p2). The N-terminal component of Pr55Gag, matrix, is required for targeting Gag and Gag-Pol polyprotein precursors to the host cell membrane prior to viral assembly (Yuan *et al.*, 1993; Zhou *et al.*, 1994). Capsid protein forms the core of the viral particle, but it is also important for viral infectivity, possibly due to role in viral uncoating in association with cyclophilin A (Luban, 1996). NC coats the viral RNA genome within the virion. It binds to the packaging signal ( $\psi$ -site), typically located in the region of the 5' RNA of the *gag* initiation codon (Clever *et al.*, 1995), and targets the RNA into the assembling virion. The very C-terminal portion of Pr55Gag polyprotein encodes for p6, which is important for the incorporation of the accessory protein Vpr during viral assembly (Kondo *et al.*, 1995; Paxton *et al.*, 1993).

The envelope (Env) glycoproteins are also synthesized as a polyprotein precursor, gp160. The precursor protein is processed by a cellular protease during Env trafficking to the cell surface, resulting in the generation of the surface and transmembrane Env glycoproteins gp120 and gp41, respectively. Gp120 contains the determinants that interact with receptor and coreceptor on the target cell surface, while gp41 anchors the gp120/gp41 complex in the membrane. In addition, gp41 contains domains that are critical for catalyzing the membrane fusion between viral and host lipid membranes during virus entry (reviewed by Freed and Martin, 1995). The newly synthesized and cleaved gp120 and gp41 are occasionally retained at the endoplasmic reticulum (ER) by interactions with CD4 molecules (Kimura *et al.*, 1994). The accessory protein Vpu (viral protein U) promotes CD4 degradation in these complexes (Willey *et al.*, 1992), allowing the Env proteins to be transported to the cell surface for virion assembly.

The regulatory enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) encoded by the *pol* gene are initially synthesized as part of a large polyprotein precursor, Gag-Pol, whose synthesis results from a frameshifting event during Gag translation. Individual *pol*-encoded proteins PR, RT, and IN are then cleaved from Gag-Pol by the viral protease. Functionally, RT is required to yield duplex DNA from the viral RNA genome for its integration into the host chromosome. RT catalyzes both RNA- and DNA-dependent DNA polymerization, and it contains an RNase H domain that cleaves the RNA portion from RNA-DNA hybrids that are formed during the reaction (Prasad and Goff, 1990). After reverse transcription, the integrase catalyzes the removal of 3' nucleotides from each newly formed DNA strand as well as their covalent joining to the target genome. IN is also involved in the removal of viral DNA 5' nucleotides and joining it to the 3' of the host target sequence (Hindmarsh and Leis, 1999). Other regulatory proteins, specifically Tat

(transactivator protein) and Rev (regulator of viral protein expression), enhance viral protein expression: Tat is critical for transcription from the HIV-1 long terminal repeat (LTR), and Rev plays a central role in transporting viral RNAs from the nucleus to the cytoplasm. Their functions are further discussed in the next chapter in the context of viral replication.

Besides Vpu, Vif (viral infectivity factor), Vpr (viral protein R) and Nef have also been termed “accessory” or “auxiliary” proteins to reflect the fact that they are not uniformly required for virus replication. Vpr transports the viral nucleoprotein complexes to the host cell nucleus after the viral entry and its “uncoating” in the cytoplasm, whereas Vif contributes to the high infectivity of the maturing virions (Malim and Emerman, 2008). However, the mechanism of Vif function is poorly defined. The role of accessory protein Nef in viral pathogenesis is discussed in more detail in chapter 2.



## 1.2. HIV replication

HIV-1 replication is an ordered process, where most of the events proceed in consecutive and stepwise manner. The viral cycle initiates when the Env gp120 binds to the CD4 surface molecules present on the CD4(+) HIV target cells. However, CD4 alone is not enough for membrane fusion and subsequent viral entry; in addition, entry requires a chemokine receptor that functions as a coreceptor during virus binding. Two chemokine receptors, the  $\alpha$ -chemokine receptor CXCR4 and the  $\beta$ -chemokine receptor CCR5 are HIV coreceptors (Berger *et al.*, 1999). The viruses replicating in macrophages (M-tropic) are referred to as R5 isolates, and

strains that infect mainly T cells (T-tropic), are called X4 viruses. The R5 isolates are usually predominant during the early phases of infection, but X4 isolates become more common as the disease progresses.

After viral entry mediated by interactions of viral surface glycoproteins and receptors on the host cell membrane, the “uncoating” of virus follows. Removal of the viral lipid bilayer results in the formation of a large nucleoprotein complex (also referred to as preintegration complex, PIC), where MA, RT, IN, and Vpr proteins remain associated with the viral genome (Bukrinsky *et al.*, 1993). The viral RNA genome is converted into double-stranded DNA in the next step of reverse transcription, and is subsequently transported to the nucleus as part of the PIC. Following nuclear import, IN catalyzes the stepwise insertion process of the linear, double-stranded viral DNA into the host cell chromosome (Kulkosky and Skalka, 1994). The integrated provirus can now serve as the template for the synthesis of the viral RNAs that will encode all the HIV-1 proteins used to direct replication of new virus. The basal transcriptional activity from the HIV-1 LTR is low but can be markedly increased by the transcriptional activator protein Tat and associated transcriptional coactivators (Kiernan *et al.*, 1999; Mujtaba *et al.*, 2002; Ott *et al.*, 1999; Wei *et al.*, 1998; Zhu *et al.*, 1997).

Most cellular mRNAs are spliced prior to their transport out of the nucleus, but HIV requires unspliced RNAs for its polyprotein precursors. To overcome this problem, the HIV-1 Rev protein and the *cis*-acting RNA sequence called Rev-responsive element (RRE), present in *env* gene, function in concert to form a complex that is capable of interacting with the cellular nuclear export machinery (Pollard and Malim, 1998). Consequently, RRE-containing RNA can be transported unspliced into the cytoplasm, and Rev uses its nuclear localization signal to shuttle back to the nucleus.

With all viral proteins synthesized, the assembly process begins at the plasma membrane of an infected cell, where host cell membrane-associated HIV-1 Gag is a major player in this process. Mediated by interactions with the NC protein, the HIV  $\psi$ -site, and additional regions of the genome, the RNA genome is packed within the newly forming virion (reviewed in D'Souza and Summers, 2005). As a final step in the process of virus assembly and release, virus particles bud from the host cell plasma membrane. During or shortly after virus release from the plasma membrane, viral protease cleaves the Gag and Gag-Pol polyprotein precursors to generate the mature Gag and Pol proteins. Once the processing is initiated, the capsid protein forms a conical shell around the RNA/protein complex. Finally, a series of structural rearrangements leads to virion maturation (reviewed in Ganser-Pornillos *et al.*, 2008). Failed virion maturation results in the loss of infectivity.

## 2. The Nef protein

Nef is a small, 27-34-kDa, protein unique to primate lentiviruses. Structurally, HIV-1 Nef consists of an N-terminal arm of approximately 70 residues followed by a well-conserved core domain (aa 71-203 in NL4-3 Nef) (Geyer *et al.*, 1999; Grzesiek *et al.*, 1996). The high conservation of the core residues implies that the core domains of HIV-1/2 and SIV Nef adopt similar tertiary structures. Within the core domain is the flexible loop region (aa 148-178), which possesses several binding motifs. HIV-2 and SIV Nefs have an additional 10-30 amino acid C-terminal stretch.

When first characterized, this protein was named Nef (negative factor), because it was expressed during the early phase of infection and was described to decrease the virus production by functioning as a transcriptional silencer (Ahmad and Venkatesan, 1988; Niederman *et al.*, 1989). Later, however, the general idea of its functions has switched from gene silencing to boosting viral pathogenesis. Despite being dispensable for replication of HIV *in vitro*, Nef is a critical factor for AIDS pathogenesis, but the underlying molecular mechanisms behind this have remained elusive. Research has also been complicated by the ability of Nef-defective viruses to replicate in transformed cells most often used for research purposes. Nef protein does not possess any intrinsic enzymatic activity, but it interacts with many endogenous host cell proteins and can thus modulate cell functions and intracellular signaling cascades to create a beneficial environment for the virus (Figure 2., p. 20). The daunting multiplicity of Nef functions has been an obstacle in drug development, as it is unlikely that any drug will block all of them. Therefore, it would be of crucial importance to identify which Nef functions are most relevant for HIV pathogenesis.

Animal models are a feasible tool to study the functional effects of a foreign gene product introduced to the host genome. The consequences of Nef expression for the host cell have been studied *in vivo* both in murine and primate models. Studies on primates other than humans or chimps usually use SIV as the infectious agent. However, a great proportion of primates, such as macaques, that are infected with SIV remain without symptoms which makes the SIV model suitable for studying the implications of a chronic infection (Daniel *et al.*, 1987). The importance of Nef protein in viral pathogenesis was first addressed in macaques infected with SIV that contained non-functional *nef* gene with a premature stop codon (Kestler *et al.*, 1991). Soon after infection, Nef sequences had reverted back to functional genotypes, and the revertants with a coding codon became dominant, indicating the importance of a functional SIV Nef for pathogenesis. Notably however, the artificially introduced stop codon did not alter the virus replication in cultured cells (Kestler *et al.*, 1991). From this point on, Nef was noted as an important factor for HIV pathogenicity and also as a possible candidate for drug targeting. These early



findings were followed by other primate studies using either viruses with a 12-bp deleted Nef (Whatmore *et al.*, 1995) or PAK-binding defective Nef mutant viruses (Sawai *et al.*, 1996) as infectious agents. In both cases, the introduced mutant *nef* genes were soon reverted: The 12-bp Nef deletion was repaired *in vivo* by sequence duplication and its evolution was continued until the repair was almost indistinguishable from wild type sequence. Similarly, in macaques that were infected with Nef-mutated SIV, the isolation of recovered viruses from these animals revealed that mutant viruses rapidly reverted to functional Nef sequence. In contrast to these findings however, another study reported that a minority of adult and most infant macaques progressed to simian AIDS even after infection with *nef*-defective SIV strains (Baba *et al.*, 1999). Thus, Nef is not an absolute requirement but rather a strong accelerator for progression of simian immunodeficiency. An important functional site in Nef has been mapped to its proline-rich SH3-binding domain PxxP (where P is a proline and x any amino acid residue). Inoculation of macaques with proline-mutated SIVmac239-P104/107A viral clone reverted back to PxxP genotype within 8 weeks after viral infection (Khan *et al.*, 1998), revealing selective pressure for functional SH3-binding during HIV pathogenesis.

In cohort studies of HIV-infected patients, approximately five percent of participants are long-term non-progressors (LTNP) or slow progressors (SP), i.e. individuals who have been infected for over ten years and remain asymptomatic without receiving any anti-HIV treatments. Characteristically, LTNPs usually show stable CD4+ T cells counts and low viral loads (Rodes *et al.*, 2004). Several LTNP- and SP individuals with HIV-1 infection carry a functionally defective *nef* gene (Alexander *et al.*, 2000; Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995; Mariani *et al.*, 1996; Salvi *et al.*, 1998). A well-studied patient group is Sydney Blood Bank cohort, consisting of eight blood transfusion recipients infected with *nef*-attenuated HIV-1 acquired from a single donor. Despite the single source of infection, the outcome of infection has been unique in each individual (Birch *et al.*, 2001; Churchill *et al.*, 2006; Verity *et al.*, 2007). Furthermore, despite deletions in Nef sequence, declining CD4 counts have been observed from an LTNP patient, suggesting that Nef alone is not responsible for lowering CD4 cell counts (Greenough *et al.*, 1999). With respect to drug development, these results rule out the possibility of using an attenuated virus vaccine approach that would be based solely on deleting Nef from the virus.

The high mutational rate of HIV makes the Nef sequence also highly variable. A comparative study on the frequency of disrupted *nef* genes and specific amino acid substitutions in Nef protein of patients with long-term non-progressing disease and individuals with progressing infection revealed that some amino acid changes correlated with low CD4(+) T cell counts and the high viral load (Kirchhoff *et al.*, 1999). Variations of T15, N51, H102, L170, and E182 were frequently observed among non-progressors, whereas an additional N-terminal PxxP-motif, A15, R39, T51, T157, C163, N169, Q170 and M182 were the residues most frequently mutated

in progressors. These findings suggested that specific changes in Nef protein can be correlated with the immune status of an individual patient. Moreover, the rarity of defective *nef* genes in HIV patients supports the idea that functional Nef is required for HIV pathogenesis.

In mouse studies, the transgenic expression of Nef was targeted into T cells through CD3 $\delta$ , CD2 or the T cell receptor (TcR)  $\beta$  chain regulatory regions (Brady *et al.*, 1993; Lindemann *et al.*, 1994; Skowronski *et al.*, 1993). All of these expression models resulted in perturbed CD4(+) T cell development in the thymus and subsequent depletion of peripheral CD4(+) T cells in Nef-expressing animals. Later, the work from Hanna *et al.* defined Nef as a determinant for AIDS-like symptoms in a murine model by reporting that expression of transgenic Nef in CD4(+) cells led to the development of a severe human AIDS-like disease in CD4C/HIV transgenic mice (Hanna *et al.*, 1998). The same group also analyzed the importance of one particular Nef-binding protein, the Src-kinase Hck, for AIDS-like symptoms in their mouse model. To this end, they used a Nef allele with a mutated Hck SH3-binding domain (PxxP). The engineered Nef-AxxA protein abolished the pathogenicity of Nef, indicating that an intact Hck-binding site, the PxxP domain, is essential for inducing of AIDS-like symptoms. However, when the importance of this interaction was tested by crossbreeding Nef-expressing HIV-1 Tg mice with the *hck/hck*-background, the pathological phenotype was only slightly delayed in the absence of Hck, in contrast to the results obtained with the Nef-AxxA transgene, thus implicating Hck as an important but not an essential effector of Nef in the HIV pathogenesis (Hanna *et al.*, 2001). A later introduction of both laboratory-adapted and patient-derived Nef alleles into transgenic mice resulted in markedly different pathogenicities, suggesting that the pathogenic capacity of a particular allele does not directly correlate with its virulence (Priceputu *et al.*, 2007). In summary, *in vivo* data obtained from mice, primates and humans alike indicate Nef as a central, but not the sole determinant for disease progression.

### 3. Cellular functions of Nef

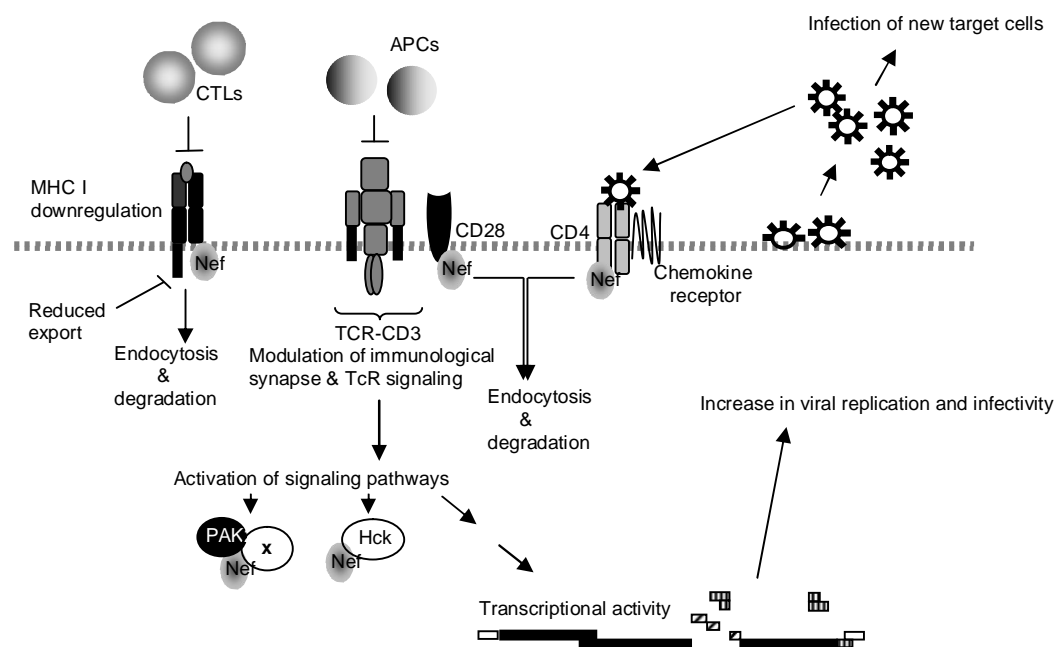
#### 3.1. Viral replication and enhanced viral infectivity

After the first dispute of the role of Nef in HIV replication, *in vitro* studies found that the allelic variants of Nef could exhibit different characteristics during viral replication (Terwilliger *et al.*, 1991). In addition, the origin of the Nef protein was considered to cause differential functions: transfer of a laboratory-adapted isolate HIV<sub>BRU</sub> Nef into CD4 (+) cells by retroviral transduction did not alter cell surface marker expression, proliferation or cytokine expression (Schwartz *et al.*, 1992). In contrast, *nef* genes isolated from freshly collected peripheral blood mononuclear cells of infected patients could accelerate virus production in primary human lymphocytes but not in T cell lines (de Ronde *et al.*, 1992).

Nef is not an essential factor for virus replication *in vitro*, but it markedly enhances virus spreading in primary cells (Miller *et al.*, 1994; Spina *et al.*, 1994). Nef also enhances the intrinsic activity of progeny virions independently of CD4 down-regulation, but requires the presence of Nef in virus-producing cells (Aiken and Trono, 1995; Chowes *et al.*, 1994; Chowes *et al.*, 1995; Miller *et al.*, 1994; Pandori *et al.*, 1996). Virions are endocytosed via clathrin-coated pits, and both clathrin and an essential guanosine triphosphatase (GTPase) of clathrin-mediated endocytosis, dynamin, are essential for Nef-dependent enhancement of infectivity (Pizzato *et al.*, 2007). Furthermore, the route of virus entry may affect infectivity enhancement as Nef is unnecessary for infectivity of HIV virions with envelopes that mediate entry through endocytic compartments (Aiken, 1997; Chazal *et al.*, 2001).

Small amounts of Nef are taken up into assembling virions (Pandori *et al.*, 1996; Welker *et al.*, 1998), but Nef uptake into the virions is not required for increased infectivity (Fackler *et al.*, 2006). The role of Nef in enhancement of HIV infectivity has been addressed to reduced susceptibility of virions to proteasomal degradation in target cells (Qi and Aiken, 2007), enhancement of cytoplasmic delivery of virions by increased entry of HIV-1 (Schaeffer *et al.*, 2001), increased viral budding from the lipid rafts with concomitant Nef-mediated alterations in raft lipid composition (Zheng *et al.*, 2001; Zheng *et al.*, 2003), recruitment of a signalosome promoting transcriptional derepression (Witte *et al.*, 2008; Wolf *et al.*, 2008b), or facilitated transport of the viral genome through the cortical actin network (Campbell *et al.*, 2004). However, conflicting results have been obtained using T cells that are acutely infected with Nef viruses, where Nef had no effect on segregation of the viral structural proteins. Thus, this questions whether Nef localizes viral proteins to rafts for virus particle assembly (Sol-Foulon *et al.*, 2004).

Antigen-presenting cells (APCs) can significantly increase the viral replication in T cells by providing activation signals to T cells. In coculture models of T cells and endothelial or dendritic cells, Nef boosted viral replication efficiency (Choi *et al.*, 2005a; Messmer *et al.*, 2000; Petit *et al.*, 2001). Interestingly, the increase of HIV-1 replication by Nef and Vpr-dependent mechanism can also take place in non-proliferating CD4(+) T cells that are minimally activated through their T cell receptor. This may imply a mechanism in which a reservoir of the virus is maintained and contributes to viral rebound in patients with changes in drug regimen (Choi *et al.*, 2005b).



**Figure 2.** Overview of the most essential Nef functions in infected T cells (modified from a review Kirchhoff *et al.*, 2008).

## 3.2. Downregulation of cell surface molecules

### 3.2.1. CD4

Cell surface marker CD4 is an HIV receptor required for infection. However, CD4 must be removed from the cell surface after the host cell is infected because CD4 interferes with viral replication, proper budding of progeny virions, and correct trafficking of the viral envelope (Lama *et al.*, 1999; Lundquist *et al.*, 2002; Ross *et al.*, 1999). The importance of CD4 downregulation for HIV-1 is reflected by the use of three different viral proteins, Vpu, Env and Nef, that downmodulate CD4 (reviewed in Lama, 2003). The observation that HIV-1 Nef expression could downregulate cellular surface marker CD4 was one of the earliest functions associated with Nef, and this function is a shared property of Nef isolates (Anderson *et al.*, 1993; Garcia and Miller, 1991; Garcia *et al.*, 1993; Guy *et al.*, 1987; Iafrate *et al.*, 1997; Mariani and Skowronski, 1993). The mechanism of Nef-induced CD4 downmodulation involves internalization of surface CD4 and subsequent targeting to the endosomal pathway for degradation. In line with this, Nef has been reported to localize to clathrin-coated pits and increase the number of CD4-containing pits (Foti *et al.*, 1997; Greenberg *et al.*, 1998a). The N-terminal attachment of a myristoyl, required for proper membrane targeting of Nef (Yu and Felsted, 1992), is also necessary for CD4 downregulation (Peng and Robert-Guroff, 2001).

Current data on CD4 downregulation suggests that association of Nef with the cytoplasmic tail of CD4 (Anderson *et al.*, 1993; Garcia and Miller, 1991; Grzesiek *et al.*, 1996; Hua and Cullen, 1997) connects the surface molecule with the endocytic machinery of the cell (Mangasarian *et al.*, 1997). Nef association with the heterotetrameric clathrin-associated adaptor protein (AP) complexes enhances CD4 endocytosis (Bresnahan *et al.*, 1998; Piguet *et al.*, 1998) by recruiting CD4 to adaptor proteins and subsequently targeting it to lysosomes. AP-1, AP-2 and AP-3 all bind to the canonical motif 161EXXXLL165 in the structurally flexible loop formed by residues 148-180 in Nef (Aiken *et al.*, 1994; Craig *et al.*, 1998; Erdtmann *et al.*, 2000; Janvier *et al.*, 2003). Recently, the association of Nef with AP-2 has been reported to require yet another Nef motif of two acetic residues 174(E/D)/D175 (Lindwasser *et al.*, 2008). In addition, bi-ubiquitylation of Nef on K144 is required for CD4 downregulation (Jin *et al.*, 2008), which may function as an additional sorting signal in Nef-mediated receptor endocytosis or trafficking.

The capacity of Nef to downregulate CD4 has been correlated with its association with human thioesterase II (hTE) (Liu *et al.*, 1997). Like so many other Nef interactions, the Nef-hTE interaction has been mapped to residues D108, L112, F121, P122 and D123 within the 'hydrophobic patch' in the Nef core domain, and

mutations to these amino acids also abolished the capacity of Nef to downregulate CD4 (Cohen *et al.*, 2000; Liu *et al.*, 2000). A more detailed understanding of the Nef-hTE complex and its connection to Nef-mediated downregulation of CD4 is lacking. However, the Nef-hTE complex has been shown to localize to peroxisomes, where thioesterase has been suggested to participate in fatty acid oxidation and lipid metabolism.

Data obtained from an LTNP patient infected by an HIV-1 with a uniquely defective *nef* gene, containing a small deletion of 36-bp and a compensating 33-bp duplication, suggested that inhibition of CD4 downregulation could delay HIV pathogenesis (Carl *et al.*, 2000). *In vitro* studies demonstrated that the deletion itself led to inactivation of CD4 and MHC I downregulation, inhibition of infectivity, and to partial destabilization of the protein. This implies that Nef-induced CD4 downregulation could be a target for therapeutical intervention.

### 3.2.2. MHC class I and II

Another surface molecule downmodulated by Nef is MHC I (Akari *et al.*, 2000; Le Gall *et al.*, 1997; Le Gall *et al.*, 2000; Schwartz *et al.*, 1996). After infection, the early expression of Nef and subsequent sequestration of MHC I from the surface of an infected cell can provide cell with a means to evade destruction by the immune system once active viral replication initiates. In favor of this, Nef expression reduces the susceptibility of HIV-infected cells to cytotoxic T cell and NK cell-mediated killing *in vitro* (Cohen *et al.*, 1999; Collins *et al.*, 1998; Lewis *et al.*, 2008; Yang *et al.*, 2002). Rather early on, it was noted that Nef-induced CD4 and MHC-I downregulation constituted genetically and mechanistically separate entities. Nef was shown to induce targeting of MHC I to the *trans*-Golgi network (TGN) and to the peripheral vesicles positive for endosomal markers (Le Gall *et al.*, 1998; Schwartz *et al.*, 1996). Mutations in the Nef myristoylation site at G2 residue and the N-terminal alpha-helical region, as well as in the polyproline and acidic motifs (EEEE65) have been found to selectively affect MHC I downmodulation (Greenberg *et al.*, 1998b; Mangasarian *et al.*, 1999; Peng and Robert-Guroff, 2001).

Studies that analyzed MHC I downregulation have presented two different pathways utilized by Nef to reduce MHC I expression on the cell surface: (i) the endocytosis of MHC I from the plasma membrane to the TGN in PACS-1 (phosphofurin acidic cluster sorting protein-1), AP-1 and clathrin-dependent manner, and (ii) binding of AP-1 to the cytoplasmic tail of MHC I to re-route it from the TGN to lysosomes.

Interaction of Nef with PACS-1 was initially shown by Piguet *et al.* as a requirement for MHC I downmodulation by Nef (Piguet *et al.*, 2000). Later, the Nef/PACS-1 interaction was reported to lead to hijacking of the ARF6 (ADP-ribosylation factor

6) endocytic pathway in the phosphoinositide-3-kinase-dependent process, which ultimately led to the retention of MHC I in the TGN (Blagoveshchenskaya *et al.*, 2002). This cascade was dependent on two Nef motifs; the acidic region EEEE65 required for binding to the cytosolic sorting protein PACS-1 and the proline domain PxxP75 important for Nef association with Src-family tyrosine kinase (SFK) (Lee *et al.*, 1995; Piguet *et al.*, 2000). Recent studies have elucidated this cascade in further detail, and the EEEE65 motif has been shown to enable Nef binding to the sorting protein PACS-2 which targets Nef to the perinuclear region (Hung *et al.*, 2007). There, PACS-2 is disengaged and Nef binds via its PxxP domain to a *trans*-Golgi localized SFK. The Nef-SFK interaction is followed by binding and subsequent tyrosine phosphorylation of the tyrosine kinase ZAP-70 (Zeta chain associated protein 70 kDa). Activation of ZAP-70 initiates signaling that eventually leads to increased activation of GTPase ARF6, thus increasing the endocytosis rate of MHC I. This updated model does not explain, however, the direct interaction found between Nef, AP-1 and cytoplasmic tail of MHC I recently reported by other groups (Noviello *et al.*, 2008; Wonderlich *et al.*, 2008). According to their data, the Nef-MHC I complex recruits the clathrin adaptor protein complex AP-1, which targets MHC I from the TGN to the endo-lysosomal network, where it is degraded. Recruitment of AP-1 is dependent on Met20 residue in the N-terminal helical domain of HIV-1 Nef, and a tyrosine residue in the cytoplasmic tail of MHC I (Le Gall *et al.*, 1998; Roeth *et al.*, 2004). Because MHC I cytoplasmic tail does not bind directly to AP-1, Nef has been suggested to serve as a facilitator in this complex. In support of the latter model, Baugh *et al.* have reported that association of Nef with PACS was not mediated by the EEEE65 stretch; instead, the Nef domain plays a stabilizing role in the complex formed by Nef, AP-1, and the cytoplasmic domain of MHC I (Baugh *et al.*, 2008). To date, the final consolidation of these two models remains unclear. However, a recent study from Schaefer *et al.* (Schaefer *et al.*, 2008) has proposed that despite differences in intracellular trafficking, both MHC I and CD4 eventually end up in similar Rab7(+) lysosomal vesicles due to the interplay of Nef and Nef-interacting protein,  $\beta$ -COP (Benichou *et al.*, 1994).

Later, Nef was shown to downregulate MHC class II (MHC II) surface expression and thus affect the antigen presentation by MHC II (Stumptner-Cuvelette *et al.*, 2001). MHC II is expressed on APCs, such as dendritic cells, macrophages, and activated CD4(+) T cells. The activation of T helper (Th) cells by APCs is necessary for the proper humoral and cellular anti-HIV immune response (Brander and Walker, 1999; Gandhi and Walker, 2002). Furthermore, Nef has been reported to upregulate the MHC II-associated invariant chain, Ii (Schindler *et al.*, 2003). The stable expression of Ii prevents antigen presentation and may therefore contribute to the impaired Th responses observed in AIDS patients (Betts *et al.*, 2001; Roche *et al.*, 1992; Stumptner-Cuvelette and Benaroch, 2002). Ii upregulation may also provide an advantage for viral replication in SIV-infected macaques (Schindler *et al.*, 2004). The physiological relevance of Ii upregulation is supported by the finding

that some Nef alleles found from LTNP patients are deficient in upregulation of Ii (Schindler *et al.*, 2003). However, despite the conserved domain structure of Nef among different clades of HIV, clade C and F Nef alleles have recently been reported impaired for Ii upregulation (Turk *et al.*, 2009).

### 3.2.3. CD80 and CD86

Surface molecules CD80 and CD86 provide the costimulatory signals necessary for proper T cell activation upon APC contact. Nef downregulates CD80 and CD86 via dynamin- and clathrin-independent pathway (Chaudhry *et al.*, 2005). However, the Rab11-dependent endocytic machinery targeting CD80 and CD86 to Golgi does not fully resemble any other described receptor internalization mechanism affected by Nef (Chaudhry *et al.*, 2007; Chaudhry *et al.*, 2008). Functionally, sequestration of costimulatory molecules from the cell surface may postpone the activation of naïve T cells and the onset of T cell response in the early stages of HIV infection, thus providing the virus with prolonged time for propagation.

### 3.2.4. CD3 and CD28

In addition to CD4 and MHC I, some Nef alleles can downregulate CD28 and even CD3, the T cell receptor molecule. Efficient downregulation of these molecules is characteristic of HIV-2 and SIV Nef alleles (Bell *et al.*, 1998; Bell *et al.*, 2001; Swigut *et al.*, 2001; Swigut *et al.*, 2003; Wildum *et al.*, 2006). CD28 is a major costimulatory factor of T cell activation and crucial for normal antigen-specific T cell responses. In infected cells, its sequestration from the cell surface may suppress the immune response and cause anergy. Both HIV and SIVmac Nef proteins have been shown to interact with CD28, and the downregulatory mechanism occurs through accelerated endocytosis via the AP-2 clathrin adaptor pathway (Swigut *et al.*, 2001). The importance of CD28 downregulatory function *in vivo* has been demonstrated by a selective mutation H196Q in SIV Nef that disrupts downregulation of CD28 (Bell *et al.*, 2001). This mutation slowly reverts in infected rhesus macaques, supporting the selective advantage gained by CD28 modulation (Schindler *et al.*, 2004; Whatmore *et al.*, 1995).

In contrast to the severe pathologic manifestations of HIV-1 infection, many simian lentiviruses do not induce the pathological effects in their natural hosts. The downregulatory effect of CD3 by SIV, but not by HIV, has been suggested by Schindler *et al.* to be an adaptive property of SIV to limit the viral pathogenicity (Schindler *et al.*, 2006). According to their hypothesis, primate infection with the virus encoding Nef, which downregulates CD3, prevents activation of infected T cells. Subsequently, activation-induced T cell death is prevented. In contrast, HIV-1



does not downregulate CD3 in infected humans enabling the hyperactivation of T cells, collapse of the immune system and progression to AIDS.

However, exceptions to the rule of CD3 downregulation by SIV (but not by HIV-1) exist. Despite its name, HIV-2 is related to SIV, possessing reduced pathogenicity as compared to HIV-1. However, progress to AIDS can occur (Brun-Vezinet *et al.*, 1987). HIV-2 downregulates CD3 which is in concert with the less pathogenic properties of both SIV and HIV-2 (Munch *et al.*, 2005). In contrast with this model are the results obtained from patients who were infected with different strains of HIV-2 that progressed to AIDS and who all had their CD3 downregulated by Nef (Munch *et al.*, 2005). Similarly, rhesus macaques infected with HIV-2 have manifested a pathogenic phenotype, although not consistently (Dormont *et al.*, 1989; Stahl-Hennig *et al.*, 1990).

### **3.2.5. Chemokine receptors**

Chemokine receptors mediate cell responses to chemokines, a family of proteins that regulate leukocyte migration and activation (reviewed in Moser and Loetscher, 2001; Stein and Nombela-Arrieta, 2005). Active secretion of chemokines controls homing and recruitment of leukocytes into tissues, and the ordered movement of leukocytes constitutes an essential function for the development and maturation of the immune response.

Chemokine receptors CCR5 and CXCR4 function as coreceptors for entry of HIV and SIV. *In vivo*-transmitted HIV-1 strains generally prefer CCR5 (R5 viruses), but strains that use CXCR4 (X4 viruses) as their entry coreceptor emerge in approximately 50 % of late stage AIDS patients (review in Philpott, 2003). X4 viruses are associated with a rapid decline in CD4(+) cell counts and progression of the disease. Strong genetic evidence on the importance of this functional coreceptor in HIV-1 is provided by the naturally occurring 32 bp-deletion mutant of CCR5 (CCR5 $\Delta$ 32), which generates a non-functional receptor that does not support viral entry of either R5 or dual tropic viruses (Samson *et al.*, 1996). Taking advantage of the requirement of coreceptor in HIV-1 infection, CCR5 receptor ligands are currently considered as endogenous anti-virals and potential inhibitors of viral entry (Lusso, 2006).

HIV-1 Nef has been implicated in the downregulation of several chemokine receptors, including the viral coreceptors CCR5 and CXCR4 (Hrecka *et al.*, 2005; Michel *et al.*, 2005; Michel *et al.*, 2006). This downregulatory function is a shared property among HIV-1 and -2 Nefs, as well as SIV alleles (Michel *et al.*, 2005), yet many HIV-2 and SIV alleles are more efficient in downregulation of CXCR4 than HIV-1 Nefs (Hrecka *et al.*, 2005; Wildum *et al.*, 2006). In concert, some SIV Nef

alleles also strongly inhibit lymphocyte chemotaxis towards the CXCR4 ligand chemokine SDF-1 $\alpha$ . Although weaker than SIV Nefs, HIV-1 alleles also possess the ability to inhibit chemotaxis to SDF-1 $\alpha$  (Choe *et al.*, 2002), reportedly by activating Rac1/2 signaling via the bipartite GEF DOCK2/ELMO1 (Hrecka *et al.*, 2005; Janardhan *et al.*, 2004). Disruption of the functional PxxP domain or Nef residue R106 which is associated with PAK2 interaction, reduced the ability to inhibit migration (Lee *et al.*, 2008). Because no PAK protein was detected during the initial mass spectrometric analysis of Nef-associated proteins by Janardhan and colleagues (Janardhan *et al.*, 2004), the role of PAK in Nef-affected chemokine signaling remains obscure. Despite an efficient downregulatory effect on ectopically expressed chemokine receptors on cultured cells, relatively modest effects on chemokine receptor expression in HIV-1 infected human cell lines and in PBMC have been observed (Michel *et al.*, 2005; Michel *et al.*, 2006; Wildum *et al.*, 2006).

### **3.3. Modulation of cellular signaling by Nef**

#### **3.3.1. Nef and Src-family tyrosine kinases**

The first interactions mediated by the proline-rich PxxP-motif in Nef that were characterized were the interactions with SFKs Hck and Lyn (Saksela *et al.*, 1995). Year later, the role of the PxxP domain in the Nef-Hck interaction was confirmed by studies utilizing Nef/SH3 cocrystals and NMR of Nef (Grzesiek *et al.*, 1996; Lee *et al.*, 1996). Besides Hck, Nef interactions with SFKs Lck, Fyn and Src have also been reported (Arold *et al.*, 1997; Arold *et al.*, 1998; Cheng *et al.*, 1999; Collette *et al.*, 1996; Dutartre *et al.*, 1998; Greenway *et al.*, 1996; Greenway *et al.*, 1999; Lang *et al.*, 1997; Lee *et al.*, 1995). However, interactions with Fyn or Lck are clearly weaker than the association of Nef with Hck or Lyn (Saksela *et al.*, 1995). The interactions of Nef alleles with SFK SH3-domains are reported to be dependent on the viral origin of Nef; the HIV-2 and SIV Nef proteins primarily target Fyn and Src, while the primary association of HIV-1 Nef is with Hck and Lyn. The altered avidity of HIV-1 versus SIV/HIV-2 Nef towards Src kinases is governed by change of three amino acids within the SH3-binding hydrophobic 'pocket' of Nef (Collette *et al.*, 2000). Later, the contribution of other residues within the hydrophobic patch of HIV-1 Nef was also reported to be important for its association with and activation of Hck (Choi and Smithgall, 2004).

Among SFKs, The primary target of HIV-1 Nef, Hck, is expressed in macrophages and neutrophils. Hck is implicated in several actin-dependent phagocytic functions involving plasma membrane and lysosome mobilization, such as phagocytosis and migration (Lowell and Berton, 1999). Hck is expressed in two isoforms, p59 and p61, the latter having an additional 21-amino acid peptide at its N-terminus. These isoforms are targeted differentially within cells; p59 is N-terminally myristoylated

and palmitoylated, whereas p61 is only myristoylated. This results in the mainly lysosomal localization of p61. On the contrary, palmitoylation of the p59 isoform results in its localization to the plasma membrane-enriched fractions, more specifically to caveolae or rafts (Carreno *et al.*, 2000; Robbins *et al.*, 1995). Stimulation of phagocytosis and subsequent Hck activation can be induced by use of appropriate chemicals or particles, such as microorganisms. Hck is involved in the signal transduction downstream of the Fc $\gamma$  receptor which mediates phagocytosis of IgG-coated particles (Durden *et al.*, 1995; Ghazizadeh *et al.*, 1994; Ibarrola *et al.*, 1997; Wang *et al.*, 1994). This internalization can be abolished by using the dominant negative form of p59Hck but not with the dominant negative p61 isoform, suggesting that both isoforms have different functions. Similarly, ectopic expression of constitutively active p59 and p61Hck triggers distinct phenotypes and functions: active p59Hck induces the formation of membrane protrusions, whereas active p61Hck triggers the formation of large F-actin-rich structures, the podosome rosettes (Carreno *et al.*, 2002; Cougoule *et al.*, 2005). Hck may also downregulate granulocyte colony-stimulating factor (G-CSF) induced proliferation of granulocytic precursor cells by binding to G-CSF receptor phosphotyrosines via its SH2 domain, leading to receptor phosphorylation and inhibition of signaling. In HIV, treatment of differentiated macrophages with Hck antisense oligonucleotides has been shown to inhibit viral replication (Komuro *et al.*, 2003). Hck activated by Nef accumulates to the Golgi apparatus (Hiyoshi *et al.*, 2008), suggesting that, in addition to stimulation of Hck activity, Nef can also modulate Hck signaling by altering where the protein is targeted.

### **3.3.2. Nef and T cell receptor signaling**

T cell activation is a hierarchical process, where different events of the cascade are performed sequentially with increasing stimulation via T cell receptor (TcR). In HIV infection, the activation status of a target cell is the major factor in dictating whether the virus can readily spread in an infected host. The main target cells of HIV, macrophages and quiescent T lymphocytes, are efficiently infected by the virus, but active replication after viral entry is blocked (Stevenson *et al.*, 1990; Stevenson, 2003; Zack *et al.*, 1990). Partial activation can, however, allow viral propagation, whereas host cell death by hyperactivation or immunological recognition by cytotoxic T cells limits virus spread. Accumulating data suggests that, with respect to T cell activation, Nef balances cell activation and prevention of apoptosis by hyperactivation.

Physiologically, stimulation of T cells by antigens via TcR leads to the clustering of TcRs, coreceptors, adhesion and signaling molecules, as well as cytoskeleton components. Together, these molecules form supramolecular activation clusters (SMACs) at T cell and APC contact site, termed the immunological synapse (IS).

The IS is a dynamic structure for ensuring efficient antigen recognition, controlled T cell activation and provision of stimulus to the APC. The SFK Lck is among the first molecules to be activated upon TcR stimulus and leads to the orchestrated recruitment of other signaling and adaptor components (reviewed in Dustin, 2009).

The ability of HIV Nef to modulate TcR signaling has been extensively studied. In infected cells, the formation of IS is impaired by a Nef-dependent mechanisms affecting actin polymerization and altered localization of Lck and N-Wasp (Haller *et al.*, 2006; Haller *et al.*, 2007). In addition, Nef increases activity of ERK MAP kinase pathway, as well as transcription factors NFAT (nuclear factor of activated T cells) and NF- $\kappa$ B (nuclear factor  $\kappa$ B) (Fortin *et al.*, 2004; Manninen *et al.*, 2001; Schragger *et al.*, 2002; Wang *et al.*, 2000). In addition to PxxP motif in Nef, myristoylation is required for increased  $\alpha$ CD3/ $\alpha$ CD28-induced IL-2 secretion via both NFAT and NF- $\kappa$ B transcription factors (Wang *et al.*, 2000).

Nef associated with several molecules involved in TcR signaling. In cells, the interaction of Nef with Vav is associated with actin rearrangement and subsequent impairment of IS (Fackler *et al.*, 1999; Quaranta *et al.*, 2003), whereas the association of Nef with inositol triphosphate receptor (IP3R) has been implicated as a TcR-independent mechanism that promotes T cell activation (Manninen and Saksela, 2002). Other Nef binding partners involved in T cell activation and proliferation include Raf-1 kinase (Hodge *et al.*, 1998) and PKC (protein kinase C) family kinases (Manninen *et al.*, 2001; Smith *et al.*, 1996; Wolf *et al.*, 2008a).

While several Nef-binding proteins in the TcR pathway are characterized, the effect of these interactions on T cell activation remains controversial. Generally, Nef is considered an enhancer of T cell signaling that sensitizes T cells for activation and leads to their increased permissiveness for HIV infection (Keppler *et al.*, 2006; Schragger and Marsh, 1999; Wang *et al.*, 2000). Findings from Fenard *et al.* show that Nef can increase the number of activated cells, but it does not affect the level of activation achieved by each individual cell (Fenard *et al.*, 2005). In addition, the transcriptional status of a cell can be similarly altered by Nef expression as through a  $\alpha$ CD3 stimulation (Simmons *et al.*, 2001). On the other hand, Nef negatively affects T cell activation by remodeling actin cytoskeleton, disrupting the formation of the immunological synapse and affecting the membrane trafficking of signaling molecules (Fackler *et al.*, 2007; Fenard *et al.*, 2005; Haller *et al.*, 2006; Haller *et al.*, 2007; Schindler *et al.*, 2006; Thoulouze *et al.*, 2006). These differences may be linked with the localization of Nef; the cytoplasmic form mainly inhibits TcR signaling, whereas membrane-targeted Nef is stimulatory (Baur *et al.*, 1994).

### 3.3.3. Association of Nef with PAK2

The initial studies on the association of Nef determined that it binds to a serine/threonine kinase that is activated by GTPases Cdc42/Rac1; this protein was characterized some years later as PAK family kinase (for more detailed description of PAKs, see chapter 4) (Lu *et al.*, 1996; Nunn and Marsh, 1996; Sawai *et al.*, 1994; Sawai *et al.*, 1995). However, the precise identity of the kinase remained elusive because two family proteins, PAK1 and PAK2, were suggested as HIV-1 Nef-associating kinases (also referred to as NAK) (Arora *et al.*, 2000; Fackler *et al.*, 2000; Renkema *et al.*, 1999). Later studies established PAK2 as the canonical NAK. The ability to associate with active PAK2 seems to be a shared feature of HIV and SIV Nef alleles (Kirchhoff *et al.*, 2004), but the capacity of Nefs to themselves activate PAK2 is more variable (Agopian *et al.*, 2007). Before the Nef-associating kinase was characterized, mutational analysis on Nef determined the critical amino acids for this interaction (Manninen *et al.*, 1998; Sawai *et al.*, 1995; Wiskerchen and Cheng-Mayer, 1996). These studies identified domains rich in proline (P69/72/75; residue numbering according to NL4-3 Nef) and arginine (R105/R106) as crucial determinants for NAK association. The first reports also nailed down the importance of Nef myristoylation residue (G2) for association with PAK2 (Sawai *et al.*, 1995; Wiskerchen and Cheng-Mayer, 1996).

Later, similar approaches of introducing series of mutations into Nef have also been used for more extensive functional analysis (Agopian *et al.*, 2006; Fackler *et al.*, 2006). In addition to confirm the previous findings on the molecular requirements of Nef-PAK2 interaction, Agopian *et al.* recently proposed an important role for hydrophobic binding surface amino acids L85, H89, S187, R188, and F191 of Nef in PAK2 association (Agopian *et al.*, 2006). Unlike Nef mutations on G2, P72/P75 and R105/R106, which affect association with PAK2, substitution of the more recently identified residues by Agopian and colleagues did not affect other Nef functions, such as the CD4 or MHC class I downregulation. Amino acids of this putative interaction patch also reside on a different surface on the core domain than the residues critical for SH3-binding or oligomerization, suggesting that Nef uses different parts of its core domain to for PAK versus SH3-binding. In addition, parallel results have been obtained from studies using Nef from primary isolates (Foster *et al.*, 2001; Luo and Garcia, 1996). Mutations present in these Nefs (F193I and S189R) totally inhibit PAK2 association. However, S189R also decreased MHC class I downregulation, which F193I did not do, suggesting that the F193 residue specifically contributes to Nef-PAK2 interaction.

Nef myristoylation has been estimated to target approximately 10% of total cellular Nef to the plasma membrane compartment, specifically to lipid rafts (Alexander *et al.*, 2004; Wang *et al.*, 2000). Efficient localization to membranes is associated with Nef functionality, but additional domains contribute to its sustained membrane

association. In a study by Alexander *et al.*, palmitoylation of two cysteine residues has been linked to its localization to membranes (Alexander *et al.*, 2004). Additional targeting motifs are also required for membrane association and raft localization, such as a stretch of arginines in the N-terminus of Nef (Bentham *et al.*, 2006; Giese *et al.*, 2006; Welker *et al.*, 1998). Mutating the myristoylation site severely impairs Nef association with PAK2, suggesting that membrane localization is one of the prerequisites for formation of the NAK complex. This idea is further supported by the diminished association of Nef and PAK2 by mutating Nef N-terminal lysines, which are also implicated in membrane localization of Nef (Giese *et al.*, 2006). In contrast, the same arginines whose mutation reduces membrane localization of Nef do not affect PAK2 association, suggesting that lost membrane targeting of Nef *per se* may not explain loss of PAK2 association. Supporting this idea are reports on conformational change of Nef based on its location within cells, which can contribute to its differential interaction properties (Arold and Baur, 2001; Hoffmann *et al.*, 2007).

After the identification of NAK as PAK2, the Nef-PAK2 association was also studied by introducing mutations to PAK2. Mutation of the binding sites of the GEF  $\beta$ -PIX ( $\beta$ -PAK-interacting exchange factor) or the SFK (PxxP/A) did not affect Nef-PAK2 association (Renkema *et al.*, 2001), suggesting that neither Nck nor  $\beta$ -PIX plays a direct role in Nef-PAK2 association. However, mutation of the Cdc42/Rac1-interactive binding domain (CRIB) totally abolished Nef-PAK2 association underlining the importance of p21-GTPases for the association of Nef and PAK2 (Renkema *et al.*, 2001).

Functionally, the biological importance of Nef-PAK2 is not well understood. Nef-PAK2 interaction may have a role in T cell activation, and subsequent stimulation of viral replication (Alexander *et al.*, 1997; Lu *et al.*, 1996; Schragar and Marsh, 1999; Wang *et al.*, 2000; Wu and Marsh, 2001). In addition, association of Nef and PAK2 has been demonstrated to have a role in apoptotic mechanisms (Wolf *et al.*, 2001; Xu *et al.*, 1999). However, a recent study using PAK-binding deficient mutant Nef F191R demonstrated that association of Nef and PAK2 neither affects HIV-1 replication efficiency nor has an effect on T cell activation or apoptosis (Schindler *et al.*, 2007).

### 3.3.4. Role of the putative Nef dimer interface *in vitro* and *in vivo*

Several *in vitro*-studies have suggested self-assembly of Nef molecules by formation of dimers and higher oligomers. Oligomerization of Nef molecules was first reported by Kienzle *et al.*, who presented NMR and immunoblotting evidence for self-association of Nef proteins (Kienzle *et al.*, 1993). Crystallization studies suggested that the molecular contacts established between Nef molecules were formed by R105, D108, I109, L112, Y115, H116, F121, P122, and D123, the well conserved residues among HIV-1 Nef isolates (Arold *et al.*, 2000). This hydrophobic core involves a “hot spot” of Y115, F121, and P122, which is surrounded by charged amino acids R105, D108, and D123. The charge complementarity achieved by electrostatic interactions between the positively and negatively charged residues R105 and D123, respectively, may be a coherence factor that promotes Nef dimerization (Arold *et al.*, 2000).

Introducing mutations to this PDI have been shown to abolish many cellular functions of Nef. Liu *et al.* reported abolished increase in virion infectivity and MHC I down-regulation by Nef mutant D123G. In addition, mutating Nef residues D108, L112, F121, and P122 caused defective in binding to human thioesterase II and down-regulation of CD4 (Liu *et al.*, 2000). Similarly, mutations of R106A, L112R and F121R all abolished binding of Nef to PAK2 (Fackler *et al.*, 2006; Manninen *et al.*, 1998). Evidence of Nef dimerization has also been obtained from study using fluorescence complementation, where two Nef molecules fused to a regulatable dimerization domain were differently tagged with N- and C-terminal sequences of green fluorescence protein (GFP). The proximity of two non-fluorescent GFP termini brought together by dimerization of Nef molecules restored the fluorescence signal. Furthermore, this dimerization also potentiated the transformation of fibroblasts via Hck activation (Ye *et al.*, 2004). However, this study did not address the importance of the Nef PDI for the observed *in vivo* effects, neither did it rule out the possibility that the interaction of the GFP termini markedly contributed to the observed association of Nef molecules. A very recent study from the same group reported that the intact PDI is required for Nef-mediated CD4 downregulation and HIV replication (Poe and Smithgall, 2009), but it did not clarify in more detail the role of charge complementarity in Nef-PDI for these functions.

### 3.4. Nef and apoptosis

The coexistence of pathogens and their hosts leads unavoidably to an arms race where both evolve mechanisms to attack and defend. Induction of controlled cell death, apoptosis, is an ancestral host-survival mechanism upon microbial attack. During coevolution, many pathogens have developed mechanisms to promote apoptosis of host immune cells as well as to suppress apoptosis in infected cells which is required for the persistence and replication of pathogen itself. HIV infection inhibits apoptosis triggered by DNA-damaging mechanisms (Schindler *et al.*, 2005). However, HIV infection also causes apoptosis of both infected and uninfected T lymphocytes by various mechanisms, some of which are mediated by HIV-encoded proteins, e.g., Nef, Env, and Tat (reviewed in Badley *et al.*, 2000).

One major apoptotic cascade initiated by activation of cell-surface receptors is the Fas/CD95/APO-1 pathway. Nef expression in infected cells has been reported to induce both Fas receptor and Fas ligand (CD95L) expression (Xu *et al.*, 1999; Zauli *et al.*, 1999). Increased FasL may protect infected cells from apoptosis by virus-specific cytotoxic T cells, which express Fas (Mitra *et al.*, 1996; Xu *et al.*, 1997).

To repress host cell apoptosis, Nef has also evolved the ability to interact with cellular kinases involved in cell death. Apoptosis signal regulating kinase ASK-1 is downstream kinase of both tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Fas-mediated proapoptotic pathways. Interaction of HIV-1 Nef with ASK-1 inhibits both of these pathways and, in addition, suppresses the activation of the downstream c-Jun amino-terminal kinase (Geleziunas *et al.*, 2001). A report by Wolf *et al.* showed that Nef mediates phosphoinositide-3-kinase (PI3K) and PAK activation, which leads to phosphorylation and subsequent inactivation of a proapoptotic Bcl-2 family protein Bad and consequently increases anti-apoptotic Bcl-2 and enhances cell survival (Wolf *et al.*, 2001). These seemingly opposing mechanisms by Nef have been described as the “armor and sword” of cell (Ameisen, 2001) because they provide protection not only from endogenous and exogenous death signals, but also help to attack the immune cells.



#### 4. p21-activated kinases

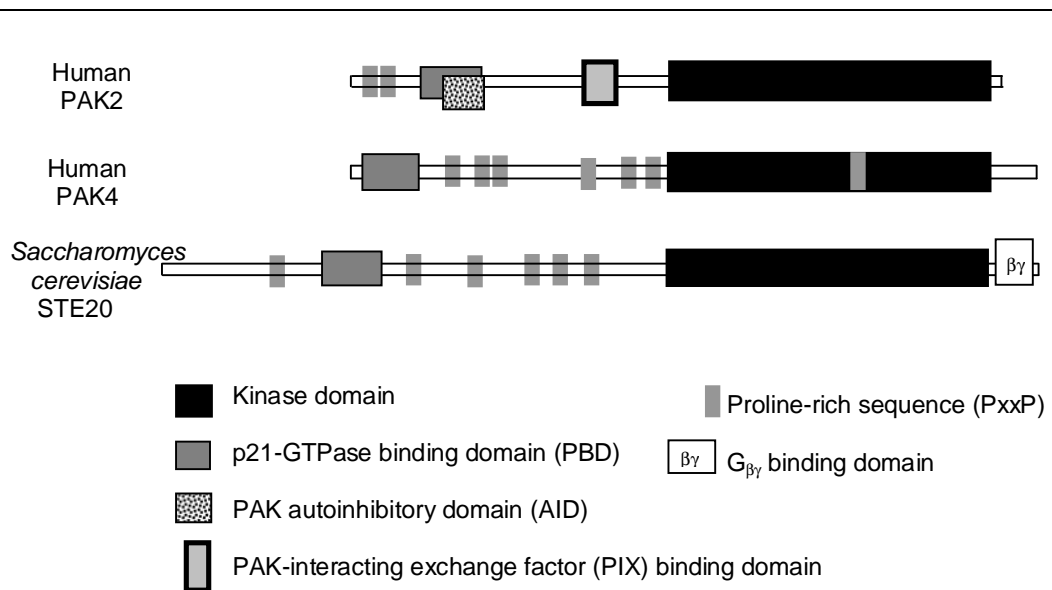
Protein kinases are an essential group of proteins in relaying signals from outside the cell to control complex intracellular processes that lead to changes in cell metabolism, migration, or proliferation. An essential theme in signal transduction within a cell is the controlled formation and release of protein complexes. Signals received from the cellular environment induce changes in protein-protein associations that relocalize proteins within the cell and modulate their activities. Kinases possess a key role in signal transduction by acutely and reversibly phosphorylating their downstream target proteins and, thus, relaying the signal. To date, more than five hundred putative human protein kinase genes have been identified (Johnson and Hunter, 2005; Manning *et al.*, 2002), and *in vivo* studies suggest that up to one-third of all cellular proteins are covalently modified by phosphorylation (Sefton, 2001). Furthermore, mounting evidence points to the key role of kinases in numerous diseases, from cancers to immunological aberrations, making them an attractive focus of drug design (Grant, 2008; Zhang *et al.*, 2009).

The PAK family is a highly conserved group of serine/threonine kinases. Nearly all eukaryotic organisms encode one or more *PAK* genes, indicating their common origin and importance in the cell. The first characterized PAK family member was found in screens for binding partners of Rac1 and Cdc42 GTPases in the rat brain (Manser *et al.*, 1994) and was an ortholog of the budding yeast *Saccharomyces cerevisiae* kinase Ste20 (Leberer *et al.*, 1992; Ramer and Davis, 1993).

In higher eukaryotes, the PAK family consists of six members, PAK1-6, each encoded by a distinct gene. PAKs are divided into two subfamilies: group A (PAK1-3) and group B (PAK4-6) (reviewed in Arias-Romero and Chernoff, 2008). The overall structure of all PAKs is similar, consisting of an N-terminal regulatory domain and a C-terminal kinase domain. However, PAKs differ in their primary structure, with most homology in the C-terminal structures, and the least homologous regions in the N-termini. Group A PAKs typically have N-terminal proline-rich (PxxP) motifs that mediate association with several SH3-domain containing proteins, a p21-binding domain (PBD; aa 67-113 in PAK1), and a C-terminal kinase domain. Group A kinases bind both Cdc42 and Rac1 and are highly activated upon binding these GTPases. The minimal binding site for p21-GTPases is referred to as the CRIB domain because it is shorter (aa 75-90 in PAK1) in comparison to the whole PBD. Group B PAKs contain a CRIB domain on their very N-terminus, and the C-terminal kinase domain. These kinases bind Cdc42 and, to a lesser extent, also Rac1 but are not markedly activated upon binding. The interaction of group B PAKs with Cdc42 induces PAK localization to different cellular compartments, suggesting regulatory differences in group A and B PAKs (Abo *et al.*, 1998; Cotteret *et al.*, 2003; Cotteret and Chernoff, 2006). In addition, a newly

characterized Cdc42-homologous GTPase Wrch-1 activates PAK1. However, it has a distinct activation mechanism in comparison to conventional Rho GTPases Rac1 and Cdc42 (Shutes *et al.*, 2004), possessing intrinsic GEF activity and intramolecular auto-inhibitory function that is regulated by the adaptor protein Grb2. Moreover, the distinct subcellular localization of Wrch-1 suggests that its role in signal modulation is different from that of Cdc42 (Berzat *et al.*, 2005).

PAK family members differ in their site of expression. PAK1 is highly expressed in brain, muscle, and spleen, although other tissues, such as the mammary gland and liver, show basal expression (Jaffer and Chernoff, 2002; Manser *et al.*, 1994). PAK2 is ubiquitously expressed in all tissues, whereas PAK3 and PAK5 expression is mostly brain-specific (Dan *et al.*, 2002; Knaus and Bokoch, 1998; Manser *et al.*, 1995; Pandey *et al.*, 2002; Teo *et al.*, 1995). PAK4 is most abundantly present in the prostate, testis and colon (Abo *et al.*, 1998; Callow *et al.*, 2002). PAK6 is also expressed in several tissues, but it is most abundant in testis, prostate, kidney, placenta, and brain (Lee *et al.*, 2002; Yang *et al.*, 2001).



**Figure 3.** PAK domain structures. Representative structures of eukaryotic group A and B PAKs and *Saccharomyces cerevisiae* Ste20 are shown. Modified from Hofmann *et al.*, 2004.

## 5. PAK functions

The most common functions of PAK family proteins are related to modulation of signaling cascades leading to transcriptional regulation, cytoskeletal remodeling and regulation of apoptotic events. The variety of PAK functions is reflected by the vast number of interaction partners, phosphorylation substrates, and mechanisms that regulate PAK activity. Due to the role of PAKs in regulating cell movement and apoptosis, increasing attention has been drawn to their importance in different types of cancer (reviewed in Kumar *et al.*, 2006). Here, some central functions of PAKs have been discussed in more detail, with an emphasis on group A proteins.

### 5.1. Cytoskeletal regulation

Cytoskeletal regulation is a central function of PAKs, with physiological relevance in cell motility, neurogenesis, angiogenesis and tumor metastasis. In T cells, actin dynamics are of key importance in T cell migration, activation and formation of the immunological synapse. The initial evidence of the role of PAK1 as a regulator in cytoskeletal dynamics came from studies showing that PAK1 was redistributed from the cytosol into cortical actin structures after cell stimulation by PDGF, insulin, wounding of a cell monolayer, or transformation by v-Src (Dharmawardhane *et al.*, 1997). These cortical actin structures included lamellae at the leading edge of the polarized cell and ruffling. Later, several studies have reported that PAK1 localizes to focal adhesions (Frost *et al.*, 1998; Manser *et al.*, 1997; Sells *et al.*, 2000). In addition, constitutively active PAK1 induces formation of filopodia, accumulation of membrane ruffles, and retraction of the trailing edge, as well as loss of actin stress fibers in cell cultures (Sells *et al.*, 1997). Signaling from active PAKs results in alterations of cytoskeleton and cell motility is often mediated further by PAK substrates that function as signal modulators. The proteins discussed below do not constitute a comprehensive list but introduce some of the downstream effectors of mainly PAK1 and PAK2 involved in cytoskeletal dynamics.

***PIX/GIT/paxillin.*** A fraction of PAKs are constitutively associated with the PIX GEFs (alternatively named COOL, cloned out of library). For binding, PIX uses a nonconventional proline-rich sequence in PAK (see Figure 3) (Bagrodia *et al.*, 1998; Manser *et al.*, 1998). The PAK-PIX complex is also associated with two serine- and threonine-phosphorylated 90- to 95-kDa proteins that were first termed PKLs (paxillin kinase linker) or CAT-1/2 (COOL-associated, tyrosine-phosphorylated) (Bagrodia *et al.*, 1999; Turner *et al.*, 1999). Later, these proteins were identified as GIT proteins (G protein-coupled receptor kinase-interactor) (Premont *et al.*, 1998; Premont *et al.*, 2000) and shown to be directly phosphorylated by PAK *in vitro* (Chong *et al.*, 2001). A focal adhesion protein, paxillin, recruits PAK to adhesion

sites via indirect binding through GIT/PKL and PIX (Brown *et al.*, 2002; Turner *et al.*, 1999; Zhao *et al.*, 2000b). In addition, PAK-GIT-PIX complexes are found in cytoplasmic vesicular compartments and in the leading edge of motile cells (Di Cesare *et al.*, 2000; Manabe *et al.*, 2002). GITs maintain the direction of cell movement, possibly due to their interaction with active Rac1 on the leading edge of the cell (Manabe *et al.*, 2002). During wound healing, PAK and PIX regulate contact inhibition (Zegers *et al.*, 2003). In T cells, the PIX-GIT complex has been implied in PAK activation and downstream transcriptional responses upon formation of the immunological synapse (Ku *et al.*, 2001; Phee *et al.*, 2005). Interestingly, a recent study has shown that PAK2-PIX-Rac1 signaling may also contribute to cell adhesion and spreading in benign tumors of the nervous system, called schwannomas (Flaiz *et al.*, 2009). These tumors are deficient in expression of PAK substrate protein merlin (Xiao *et al.*, 2002) discussed in the next paragraph.

***Nf2/merlin/schwannomin.*** The Nf2 (neurofibromatosis type2) tumor suppressor gene product merlin (also dubbed schwannomin) is related to the proteins of the band 4.1 superfamily of ezrin, radixin, and moesin, known as ERM proteins (Tsukita and Yonemura, 1997). Mutations in and loss of heterozygosity of the *Nf2* gene are involved in the pathogenesis of various sporadic tumors, such as schwannomas, meningiomas and ependydomas. All ERM proteins function as linkers between the plasma membrane and the actin cytoskeleton, and they localize to cortical actin structures near the plasma membrane such as microvilli, membrane ruffles, and lamellipodia (Tsukita and Yonemura, 1997; Tsukita *et al.*, 1997). Merlin binds to several proteins that localize to cell adhesion sites, including paxillin, focal adhesion kinase,  $\beta$ -1-integrin, and microtubules (reviewed in Scoles, 2008). The significant number of merlin interactions with proteins involved in GTPase, MAPK and PI3K pathways suggests that these signaling cascades contribute to the cellular adhesion properties regulated by merlin.

Parallel to PAK activation, merlin activity is regulated by the intramolecular binding of the N- and C-terminal domains. This binding conformationally regulates merlin by masking the binding sites for interacting proteins. Phosphorylation of merlin on the major target site S518 results in inactive protein and subsequent inhibitory effects on cell proliferation and cell cycle (Kissil *et al.*, 2003; Xiao *et al.*, 2005). In cells, merlin is phosphorylated on S518 by PKA, as well as both group I and II PAKs has been reported (Alfthan *et al.*, 2004; Kissil *et al.*, 2002; Xiao *et al.*, 2002). However, the interplay between merlin and PAK is bidirectional: merlin may inhibit PAK activity by binding to PAK1 PBD sequence and disturbing PAK recruitment to focal adhesions (Kissil *et al.*, 2003). In addition, merlin negatively regulates Rac-induced signaling (Shaw *et al.*, 2001). Notably, HIV-1 Nef has also been shown to induce merlin phosphorylation in primary T cells in a PAK activation-dependent manner (Wei *et al.*, 2005), but establishing the possible role for Nef in merlin-mediated signaling requires further study.

**LIM kinases.** The LIMK family of serine kinases consists of LIM kinases -1 and -2, which regulate actin dynamics through their ability to phosphorylate cofilin/actin depolymerizing factor (ADF) family members (for review, see Bernard, 2007). LIM kinase-1 activity was first associated with stimuli that activate Rac GTPase (Arber *et al.*, 1998; Yang *et al.*, 1998), and PAK1 was later identified as a mediator in this signaling cascade (Edwards *et al.*, 1999). In addition to PAK1, PAK2 and PAK4 also regulate LIM kinases (Dan *et al.*, 2001; Misra *et al.*, 2005). Functional LIM kinase and its downstream effector protein cofilin/ADF are required in several processes that require rapid rearrangements of actin cytoskeleton, such as microtubule dynamics (Arber *et al.*, 1998; Gorovoy *et al.*, 2005; Yang *et al.*, 1998), the cell cycle (Amano *et al.*, 2002; Sumi *et al.*, 2002) and axon growth (Tursun *et al.*, 2005). In T cells, signaling via LIMK and cofilin is required for chemokine-stimulated migration (Nishita *et al.*, 2005).

**Filamin A.** Filamins are actin-binding proteins that stabilize cytoskeletal structures and integrate the actin network to cell signaling (reviewed in Stossel *et al.*, 2001). Physiologically, they are essential for cell movement and fetal development. Filamin A was initially identified as a PAK1 binding partner by two-hybrid screen and the interaction was later confirmed by pulldown and coimmunoprecipitation assays (Vadlamudi *et al.*, 2002). The binding site of filamin A overlaps with the CRIB/PBD domain in PAK1, and filamin binding relieves autoinhibition of PAK1 and, therefore, increases the intrinsic activity of PAK1. Filamin-induced PAK1 activation also stimulates cofilin phosphorylation, possibly through LIMK-mediated signaling. Because filamin itself can also bind Cdc42/Rac1, it may serve both as a direct PAK activator and/or as a platform for GTPase-mediated activation (Ohta *et al.*, 1999). In a later study by Schiller *et al.* filamin may bridge a GEF protein Kalirin with PAK, resulting in lamellipodia formation through activation of PAK (Schiller *et al.*, 2005). The capacity of filamin A to interact with proteins involved in TcR signaling suggests that it has a role as a regulator of T cell function (Hayashi and Altman, 2006; Tavano *et al.*, 2006).

## **5.2. Cell death and survival**

PAK family members participate in apoptotic pathways to promote both pro- and antiapoptotic events (Strasser *et al.*, 2000). The proapoptotic PAK2 is activated in response to stimuli that results in cytoostasis or cell death as well as other types of cellular stress, such as hyperosmolarity, heat shock or UV irradiation (Chan *et al.*, 1998; Chan *et al.*, 1999; Roig and Traugh, 2001; Tang *et al.*, 1998).

After induction of apoptotic cascade, PAK2 is cleaved by activated caspases into 28-kDa N-terminal and 34kDa C-terminal fragments (Lee *et al.*, 1997; Rudel and

Bokoch, 1997; Walter *et al.*, 1998). The C-terminal cleavage product contains the complete catalytic kinase domain, which is constitutively active after proteolytic removal of the regulatory domain. This GTPase-independent activation mechanism may regulate morphological and biochemical alterations that are necessary for apoptosis. In support of this idea is the finding that transfection of active PAK2 C-terminus induces changes in cellular and nuclear morphology that promote apoptosis (Lee *et al.*, 1997). The C-terminal PAK2 fragment also activates the c-Jun N-terminal kinase pathway *in vivo* (Rudel *et al.*, 1998). Expression of a dominant negative PAK2 mutant delayed apoptosis and decreased formation of apoptotic bodies (Rudel and Bokoch, 1997).

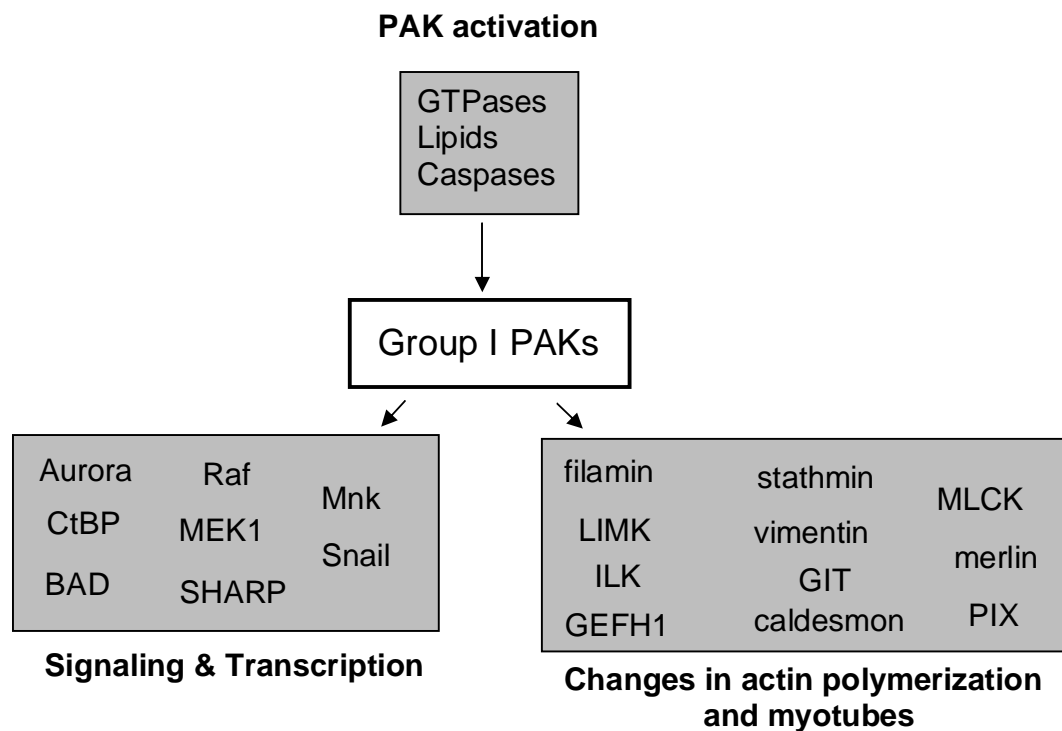
In contrast to the proapoptotic functions of PAK2, PAK1 inhibits proapoptotic events in cells. As a consequence of PAK1 activation, the death-promoting protein of the Bcl-2 family, Bad, is phosphorylated (Schurmann *et al.*, 2000; Tang *et al.*, 2000). This phosphorylation causes Bad to dissociate from Bcl-2 or Bcl-xL, freeing them to exert their antiapoptotic actions. Moreover, PAK1-mediated Bad phosphorylation has been reported to be Nef-dependent (Wolf *et al.*, 2001).

### **5.3. Cell growth and transformation**

The ability of PAKs to regulate many cellular activities that closely intertwine with transformation, such as growth factor and steroid-receptor signaling, transcription and mitosis have made PAKs an attractive target of cancer studies and therapeutical approaches. Initially, the evidence that indicated a possible role for PAK signaling in cellular transformation came from the observation that the CRIB domain of PAK1 inhibited Ras- and Rac-induced transformation of Rat-1 fibroblasts (Osada *et al.*, 1997). Further studies showed that PAK kinase activity was required for the Ras-induced transformation of fibroblast cells (Frost *et al.*, 1996; Frost *et al.*, 1997; Tang *et al.*, 1999). Later, PAK1 was determined to be essential for Rac- and Vav-induced transformation (Mira *et al.*, 2000; Sachdev *et al.*, 2002), placing PAK signaling as a downstream convergence point in transformation induced by small GTPases activated by mitogenic factors.

The yeast PAK analogue, Ste20, acts as a MAP kinase kinase kinase (MAPKKKK) in the yeast mating pathway (Dan *et al.*, 2001). This suggested that PAK may be a regulator of MAP kinase pathway. Previously, PAK-1 to -3 had been found to regulate c-Jun N-terminal kinase (JNK) and p38 MAP kinase activities (Bagrodia *et al.*, 1995a; Rudel *et al.*, 1998; Zhang *et al.*, 1995). The activatory effect of PAK on MAP/ERK kinase pathway was first presented by Lu *et al.* (Lu *et al.*, 1997), who reported that membrane targeting and subsequent activation of PAK correlated with p38, JNK, and ERK1 stimulation. Active PAK is also required for the synergistic activation of ERK by Ras and Rho GTPases (Frost *et al.*, 1996; Frost

*et al.*, 1997; Tang *et al.*, 1999). Phosphorylation of MAP/ERK S298 is critical for binding of Raf-1, and PAK2 was later identified as the kinase that phosphorylates Raf1 at Ser338, the essential regulatory site for Raf-1 activation (King *et al.*, 1998). The role of PAK-1, -2, and -3 in phosphorylation of Raf-1 Ser338 is central for a number of signaling cascades initiated by growth factors and integrins (Chaudhary *et al.*, 2000; Zang *et al.*, 2002). Later studies have identified numerous transcription factors and transcriptional coregulators (Barnes *et al.*, 2003; Orton *et al.*, 2004; Vadlamudi *et al.*, 2005; Yang *et al.*, 2005), as well as cell cycle-regulating proteins (Maroto *et al.*, 2008; Zhao *et al.*, 2005), as PAK substrates (Figure 4.) that are involved in tumor progression via regulation of gene activity and mitosis.



**Figure 4.** Overview of PAK signaling. PAK activation leads to its interaction with numerous substrate proteins contributing to cytoskeletal regulation, cell migration, signaling and transcriptional activity.

## 6. Regulation of PAK activity

### 6.1. Regulation by GTPases

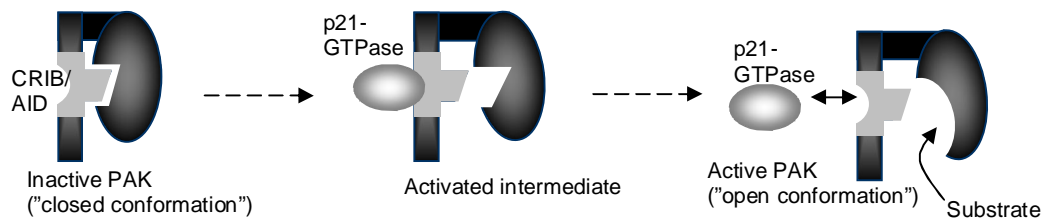
The original study that identified PAKs as GTPase-associating proteins was followed by a series of reports confirming that at the molecular level the kinase activity of PAK1 to -3 was regulated by binding of Rho GTPases (Bagrodia *et al.*, 1995b; Knaus *et al.*, 1995; Martin *et al.*, 1995). Several structural (Gizachew *et al.*, 2000; Hoffman and Cerione, 2000; Lei *et al.*, 2000; Morreale *et al.*, 2000; Thompson *et al.*, 1998) and biochemical (Benner *et al.*, 1995; Buchwald *et al.*, 2001; Chong *et al.*, 2001; Gatti *et al.*, 1999; Tu and Wigler, 1999; Yu *et al.*, 1998; Zenke *et al.*, 1999; Zhao *et al.*, 1998) studies supported a model in which the binding of GTPase disrupts the dimerization of the termini and leads to a series of conformational changes that stabilize the inhibitory switch domain. The crystal structure of PAK1 in an autoinhibited conformation was determined, and PAK1 was found to exist as a homodimer both in cells and in solution (Lei *et al.*, 2000; Parrini *et al.*, 2002; Pirruccello *et al.*, 2006). In this *trans*-inhibited conformation the N-terminal regulatory domain of one PAK molecule binds and inhibits the C-terminal catalytic domain of another molecule. The dimerization segment was mapped within the PBD/CRIB, just upstream of the autoinhibitory domain. PAK1 residues Leu107, Glu116 and Asp126 were implicated as critical residues contributing to the inhibitory interface (Lei *et al.*, 2000). A mutation introduced to any of these amino acids destroys the interaction between the inhibitory switch domain and the catalytic domain, resulting in constitutive conformational change and kinase activity.

Activation by GTPases results in autophosphorylation of specific amino acids T402, S141, and S165 (in PAK2), which are key residues for full protein kinase activation (Jung and Traugh, 2005). *In vitro* studies by Gatti *et al.* have shown that stimulation by Cdc42 induces phosphorylation at five other serine residues on PAK2 (S19, S20, S55, S192 and S197) (Gatti *et al.*, 1999). Phosphorylation of Thr402 maintains the relief of autoinhibition and full catalytic activity towards exogenous substrates (Gatti *et al.*, 1999; Yu *et al.*, 1998; Zenke *et al.*, 1999). Binding of p21-GTPase to PAK inhibits its intrinsic and GAP-stimulated GTP hydrolysis (Manser *et al.*, 1994), whereas full activation leads to the dissociation of Cdc42 and PAK2. Autophosphorylation of some N-terminal regulatory sites occurs in *cis*, but phosphorylation of the activation loop threonine (T402) is an intermolecular process that occurs by phosphorylation of one PAK kinase domain by another.

Despite the high sequence similarity in the PBD of PAKs, different PAK isoforms have different affinity for Rho GTPases. PAK1 can be activated by Rac1, Rac2, Rac3 or Cdc42 (Knaus *et al.*, 1998; Manser *et al.*, 1994; Mira *et al.*, 2000), as well



as Chp, TC10 and Wrch-1 (Aronheim *et al.*, 1998; Neudauer *et al.*, 1998; Shutes *et al.*, 2004). The amino acids affecting GTPase selectivity have been mapped mostly within the overall PBD domain (Reeder *et al.*, 2001). However, residues outside the PBD may also have an effect; the stretch of lysines (aa 66-68 in PAK1) just upstream of the CRIB domain has been implicated in efficient Rac binding and optimal stimulation of PAK kinase activity by bound GTPase (Knaus *et al.*, 1998).



**Figure 5.** Simplified model for the activation-associated conformational change of PAK2 by p21-GTPase.

## 6.2. Regulation by GTPase-independent mechanisms

While stimulation by p21-GTPases is considered the most important activation mechanism of PAKs, GTPase-independent stimuli have also been described. Initially, PAK autophosphorylation and kinase activity were reported to increase by partial protease-mediated digestion (Benner *et al.*, 1995; Roig and Traugh, 2001). This phenomenon was characterized in more detail by studies describing that PAK2 can be cleaved at a single site (aa 212 in PAK2) between the N-terminal regulatory domain and the C-terminal catalytic domain by caspases during apoptosis, generating a constitutively active PAK2 fragment (Walter *et al.*, 1998). The proteolytic activation of PAK2 allows the regulation of morphological changes that are seen in apoptotic cells (Rudel and Bokoch, 1997). The physiological consequences of this activation mechanism are discussed in more detail below in chapter *Cell death and survival*.

Association of PAK1 with an SH3-containing adaptor protein Nck and subsequent membrane localization of PAK increase its kinase activity (Lu *et al.*, 1997). The interaction of PAK with Nck has been mapped in detail to PAK sequence PxxPxRxxS21, where phosphorylation of Ser21 inhibits Nck binding (Zhao *et al.*, 2000a). In addition, increase in PAK activity has been shown by addition of a

specific membrane-targeting sequence (Daniels *et al.*, 1998). Membrane targeting of PAK may involve its interaction with lipids, e.g. sphingosine, or phosphorylation of the critical PAK1 Thr402 residue by pyruvate dehydrogenase kinase, both of which have been reported as GTPase-independent mechanisms of PAK activation (Bokoch *et al.*, 1998; King *et al.*, 2000).

Other protein kinases are involved in PAK activity modulation: Cdk5, a neuron-specific kinase, associates and phosphorylates PAK1 at Thr212. This phosphorylation, however, does not seem to significantly alter PAK kinase activity (Rashid *et al.*, 2001). Similarly, the non-receptor tyrosine kinase Etk/Bmx, tyrosine kinase Abl, as well as PI3K associate with and phosphorylate PAK1/2 (Bagheri-Yarmand *et al.*, 2001; Papakonstanti and Stournaras, 2002; Roig *et al.*, 2000). Tyrosine phosphorylated PAK1 has also been identified from a multiprotein complex in constitutively activated v-ErbB receptor transformed cells, but the protein responsible for PAK phosphorylation protein within the complex has remained uncharacterized (McManus *et al.*, 2000).

Phosphorylation of PAK is critical for its full activity, but dephosphorylation plays an equally important role in shutting off kinase activity. Two phosphatases of the same protein family, POPX1 (partner of Pix 1) and POPX2, dephosphorylate PAK1, including the key residue Thr402 (Koh *et al.*, 2002). Both of these phosphatases bind to several forms of Rac- and Cdc42-specific GEFs called PIX to form multimeric protein complexes containing PAK. The  $\alpha$ -PIX binds both PAK1 and PAK2, and to induce PAK1 activation independently, but synergistically, of Rac or Cdc42 (Feng *et al.*, 2002). In contrast to the stimulatory effect of  $\alpha$ -PIX,  $\beta$ -PIX inhibits Cdc42- and Rac-stimulated PAK activity (Bagrodia *et al.*, 1998; Feng *et al.*, 2002). In a similar manner, hPIP1, a WD-domain containing regulator protein binds PAK1 and inhibits its ability to be activated by Rac or Cdc42 (Xia *et al.*, 2001). The physiological significance of PAK negative regulation, however, remains unclear.

## **STUDY AIMS**

The association of Nef with active PAK2 kinase is common to diverse Nef proteins of both HIV-1/2 and SIV origin. Among the total cellular pool of PAK2, only a small, highly active fraction of this kinase associates with Nef, suggesting that activation of PAK2 promotes its interaction with Nef. However, the exact molecular mechanisms involved in the generation of superactive PAK2 and in the formation of NAK complex have remained elusive. PAK2 localizes to the plasma membrane upon activation. Because part of the cellular pool of Nef protein is also present in the membraneous compartment, we wanted to study in more detail the intracellular location of the NAK complex. Previously, several binding partners including Src-family kinases and p21-GTPase-activating GEF proteins, have been described for Nef. However, the contribution of SFKs or GEFs for PAK2 activity and the Nef-PAK2 complex has not been fully determined.

Detailed aims of the present study are:

- to characterize the role of Src tyrosine kinases in PAK2 activation
- to examine the molecular requirements of the Nef and PAK2 association
- to identify GEF protein partners associated with the Nef-PAK2 complex
- to study the role of self-association in Nef biological functions

## MATERIALS AND METHODS

This chapter briefly describes the main methods used. For more detailed descriptions of the methods, please refer to the original communications I-IV.

### 1. Mammalian cell culture and transfection (I-IV)

Human embryonic kidney fibroblast cells (HEK 293T) and TZM cells were cultured in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine and 1% penicillin/streptomycin (all from Invitrogen). TZM cells are a HeLa cell line that expresses  $\beta$ -galactosidase and luciferase under the control of HIV-1 long terminal repeat. The human T-cell leukemia cell line Jurkat Tag (JTag) and its derivatives JVav and BE $\alpha$ 16-3 were cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin (Invitrogen). JVav and BE $\alpha$ 16-3 cell lines lack expression of Vav1 or DOCK2 (Cao *et al.*, 2002; Sanui *et al.*, 2003), and they were kindly provided by Drs. D. Billadeau and Y. Fukui, respectively.

Transient transfections of 293T cells were done using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Jurkat Tag, JVav and BE $\alpha$ 16-3 cells were transfected by electroporation (230 V for BE $\alpha$ 16-3 or 250 V for JTag and JVav, 950  $\mu$ F,  $\infty$   $\Omega$ ) in GenePulser Xcell (BioRad) and incubated for 24-72 h depending on the cell type and construct used.

### 2. Plasmid constructs (I-IV) and RNAi oligonucleotides (III)

The expression vector for dominant active (V12) Cdc42V12 and a pEBB eukaryotic expression vector derived from pEF-BOS plasmid (Mizushima and Nagata, 1990) were kindly provided by Dr. B. Mayer (University of Connecticut, Farmington, US). The insert encoding PAK2 was cloned in pEBB with either a Myc-His-HA (hemagglutinin) multiepitope (ME) tag (Renkema *et al.*, 2001) or Myc-His (MH) tag. All PAK2 mutants (for list of mutated PAK2 derivatives, see also Table 1) were created by overlap PCR using a specific mutant primer pair and common outer primers containing restriction sites used for cloning. The PAK2 double mutants with E115K or D125R autoinhibitory mutations combined to HH (H82/85) or ISP (I74N/S75P/P77A) mutation were made by cloning the HH- or ISP-mutated N-terminal fragment to the pEBB-PAK2-ME vector containing the respective autoinhibitory mutation. As a substrate for PAK2-kinase assays (Tuazon *et al.*, 1997), three tandem copies of the 7-amino acid peptide of the Rous sarcoma

nucleocapsid (KKRKSGSL) in frame with glutathione S-transferase (GST) were cloned into the bacterial expression vector pGEX-4T-1 (Amersham Biosciences).

The NL4-3, 8161 and 13127 HIV-1 Nef alleles and SIVmac239 Nef used in communication II were a gift from Frank Kirchhoff (University of Ulm, Germany) (Kirchhoff *et al.*, 2004). SIV SYK51 Nef allele was provided by Dr. B. Hahn (University of Alabama at Birmingham, USA), and HIV-2 cbl Nef was amplified from an HIV-2-infected culture obtained through NIH AIDS Research and Reference Reagent Program. Wild type SF2 Nef and SF2 Nef mutants G2A and AxxA were a gift from Mark Harris (University of Leeds, UK). To generate Nef.GFP plasmid, the SF2 Nef allele was cloned into the pEGFP-N1 vector (ClonTech) and the expression constructs of NefF195A.GFP or NefF191I.GFP were generated using site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene). The Nef allele used in communication IV was obtained from the HIV-1 SF2 strain, which has a 4-aa duplication in its N-terminal region (residues 22-25 of the HIV-1 Nef consensus sequence), but we used the established amino acid numbering matching to the widely used strains like HXB2 and NL4-3 when discussing the mutated residues in the core domain of SF2 Nef. R105D, D123R, and R105D/D123R mutations were introduced into Nef by overlap PCR using specific mutant primers and common outer primers with restriction sites for cloning into both a pEBB vector with a C-terminal myc-tag, and also into a bicistronic vector pWPI-PP expressing IRES-GFP and a C-terminal 123-amino acid biotin acceptor sequence (PP) from *Propionibacterium shermanii* transcarboxylase obtained from PinPoint-Xa1 T-vector (Promega) (Heikkinen *et al.*, 2008). All Nef alleles and SF2 Nef mutants used in this study are also listed in Figure 6. The original expression vector pWPI was provided by Tapio Visakorpi (University of Tampere, Finland).

The full-length cDNA of *vav1* was subcloned into the pEF-BOS expression vector. The pLacZ reported plasmid was created by inserting  $\beta$ -galactosidase cDNA into pEBB. Expression plasmids for  $\beta$ -Pix.myc, Flag.DOCK2 and ELMO1.His were kindly provided by Drs. Ivan Dikic, Shinya Tanaka and Yoshinori Fukui, respectively (Nishihara *et al.*, 2002; Sanui *et al.*, 2003; Schmidt *et al.*, 2006). The cDNA of Hck isoform p61 was PCR-amplified from an IMAGE clone (<http://www.geneservice.co.uk>; ID 4855747/Accession BC014435) and subcloned into the pEBB expression vector, and a C-terminal biotinylation tag (PP) was added.

Targeted sequences by RNAi were as follows: 5'- UCU GUA UAC ACA CGG UCU GTT -3' for PAK1; 5'- AGA AGG AAC UGA UCA UUA ATT -3' for PAK2; 5'- CGU CGA GGU CAA GCA CAU UTT -3' for Vav1; 5'- AGU CCG GUC CAU AGU CAA CTT -3' for Vav2; 5'- ACC ACU GUC UGC AAU AAU ATT -3' for ELMO1, 5'- GGA ACG ACA UCU ACA UUA CTT -3' for DOCK2; 5'- GGA UGA AGU UCA AGA AUU ATT -3' for  $\beta$ -PIX; and 5'-

AGGUAGUGUAAUCGCCUUGTT -3' as a non-specific control (all from MWG-Biotech).

PAK2 mutant	Acronym	Reported functional consequences	Reference
H82/85L	CRIB	p21-GTPase-binding deficient	Zhao <i>et al.</i> , 1998
I74N/S75P/P77A	ISP	p21-GTPase-binding deficient	Zhao <i>et al.</i> , 1998
L106F	-	Conformationally autoactivated kinase	Lei <i>et al.</i> , 2000
E115K	-	Conformationally autoactivated kinase	Lei <i>et al.</i> , 2000
D125R	-	Conformationally autoactivated kinase	Lei <i>et al.</i> , 2000
Y130F Y139F Y194F	-	Disrupted phosphorylation of tyrosine residues	Renkema <i>et al.</i> , 2002
P185G/R186A	PIX	$\beta$ -PIX-binding deficient	Manser <i>et al.</i> , 1998
K278R	KD	Deficient in kinase activity ('kinase inactive')	Tang <i>et al.</i> , 1997
T402E	DA	Autoactivated kinase ('dominant active')	Sells <i>et al.</i> , 1997

**Table 1.** The PAK2 mutants used in this study.

### 3. Antibodies (I-IV)

Sheep polyclonal Nef antiserum was provided by Dr. Mark Harris (Leeds University, UK). The mouse monoclonal anti-HIV-1 Nef antibody was a gift from Professor K. Krohn (FIT Biotech, Finland). Polyclonal rabbit serum against GFP was kindly provided by Prof. Hans-Georg Kräusslich (University of Heidelberg, Germany). Anti-AU1 antibody was obtained from Biosite, and monoclonal anti-myc antibody 9E10 was purchased from either Sigma-Aldrich (used in communications I and II) or Santa Cruz Biotechnology (in comm. III). The rabbit polyclonal antibodies for ELMO1 and Vav2, as well as the anti-phosphotyrosine antibody PY20 were purchased from Santa Cruz Biotechnology. Monoclonal mouse anti-flag (M2) and anti-GFP (GFP20) were obtained from Sigma-Aldrich, and polyclonal rabbit anti-PAK1/2/3 and anti-Vav were purchased from Cell Signaling Technology. Polyclonal rabbit anti- $\beta$ -Pix and monoclonal mouse anti-His6 (BMG-His-1) were

obtained from Roche; polyclonal rabbit anti-LAT was obtained from Upstate Biotechnology; and monoclonal mouse anti-transferrin receptor (clone H68.4) was purchased from Zymed Laboratories. The secondary fluorescent antibodies and Alexa Fluor 555-conjugated cholera toxin subunit B used in cholera toxin clustering studies (comm. III) were obtained from Molecular Probes. The horseradish peroxidase-conjugated cholera toxin subunit B used in raft fractionation studies (comm. II) was purchased from Sigma-Aldrich.

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1 M[G]GKWSKRSMGGWSAIRERMRRAE - - - - - SF2
1 MGGKWSKSSVIGWPAVREERM - - - - - NL4-3
1 MGNVLGKKGKFEGWRSRVRERMRKKT - - - - - 8161
1 MGNVLTKKSQFPFGWATVREKMRRTSRRRTS - - - - - 13127
1 MGASGSKERSRSPSQGLRERLLRARGETTCGGHCSGSGGEYL HIV-2
1 MGGAISMRRSRSPSGDLRQLRLRARGETYGRLLGEEVEDGYS SIVmac
1 MGSQSSKKQPSRWDEKWRTRWVPPFGKPYSPMPFDELLRMSQ SYK51

25 PRAEPAADGAVSRD - - LEKHGAITSSNTAATNAD - - - SF2
22 -RAEPAADGAVSRD - - LEKHGAITSSNTAANNAA - - - NL4-3
25 PEPEPCAPGVGQISQE - - LAARGGIPNSYTPQNNAA - - - 8161
32 PEPEPCAPGVGQISRE - - LAARGGIPSSYTPQNNAA - - - 13127
41 QSQEESGREQRSPSYE - - GQRYQQGDFMNTPWRTPAKEGE HIV-2cb1
41 QSPGGLDKGLSSLSCE - - GQKYNQGYMNTPWWRNPAEERE SIVmac
41 P Y H E D F D R G W R S T L T E P I L D P K R D F D S G G K K W N A G D - - - SYK51

59 - - - - - C A W L E A Q E E E E V G F P V R [P Q V P] L R P M T Y K A A L D SF2
55 - - - - - C A W L E A Q E E E E V G F P V T P Q V P L R P M T Y K A A V D NL4-3
59 - - - - - L A L L E S H Q D E E V G F P V R P Q V P L R P M T F K G A F D 8161
66 - - - - - L A F L E S H Q D E E V G F P V K P Q V P L R P M T F K G A F D 13127
79 K E L Y K Q Q N R D D V D S D D D L V G V S V T P R V P L R E L T H R L A I D HIV-2cb1
79 K L A Y R K Q N M D D I D E E D D D L V G V S V R P K V P L R T M S Y K L A I D SIVmac
77 - - - - - I C H D E G D E D L V G F P V L P Q V P L R P L T Y K L A I D SYK51

91 I S H F L K E K G G L E G L I W S Q [R] R Q E I L D L W I Y H T Q G Y F P [D] W Q N SF2
87 L S H F L K E K G G L E G L I H S Q R R Q D I L D L W I Y H T Q G Y F P D W Q N NL4-3
91 L S F F L K E K G G L E G L I Y S P K R A E I L D L W V Y H T Q G F F P D W Q N 8161
98 L S F F L K E K G G L E G L I Y S H E R A E I L D L W V Y N T Q G F F P D W Q C 13127
119 V S H F I K E K G G L E G M Y Y S E R R H R I L D I Y L E K E E G I I A D W Q N HIV-2cb1
119 M S H F I K E K G G L E G I Y Y S A R R H R I L D I Y L E K E E G I I P D W Q D SIVmac
108 L S H F I K R K G G L Q G M F F C Q K R H E I L Q L Y L K N E H G V I D D I T - SYK51

131 Y T P G P G I R Y P L T F G W C F K L V P V E P E K V E E A - N E G E N N S L L SF2
127 Y T P G P G V R Y P L T F G W C Y K L V P V E P D K V E E A - N K G E N T S L L NL4-3
131 Y T P G P G T R F P L T F G W L F K L V P V S E A E A E R L G N T R E N A S L L 8161
138 Y T P G P G P R F P L T F G W L F K L V P V T E E D A E R L G N T C E R A S L L 13127
159 Y T H G P G I R Y P M F F G W L W K L V P V D V T R Q E E D - - - D G T H C L L HIV-2cb1
159 Y T S G P G I R Y P K T F G W L W K L V P V N V S D E A Q E - - - D E E H Y L M SIVmac
147 Y T S G P G T R Y P L I F G W L W E L A P N E I E G Y L V D - - - E E D T L M M SYK51

170 H P M S L H G M - E D A E K E V L V W R F D S K L A [F] H H M A R E L H P E Y Y K SF2
166 H P V S L H G M - D D P E R E V L E W R F D S R L A F H H V A R E L H P E Y F K NL4-3
171 H P A C A H G F - E D Q H K E I L M W K F D R T L G N T H V A M V T N P E L F L 8161
178 H P A C A H G Q - E D P H G E I L M W K F D R S L G S T H V A K I T N P E L F Q 13127
196 H P A Q T S R F - D D P H G E T L I W K F D P T L A H D Y K A F I L H P E E F G HIV-2cb1
196 H P A Q T S Q W - D D P W G E V L A W K F D P T L A Y T Y E A V Y R Y P E E F G SIVmac
184 H P A A G V G A S E D P H R E N L M W N F N P H L A Y T P G W E M A R Q Q L E R SYK51

209 D C . SF2
205 N C . NL4-3
210 K D . 8161
217 K D . 13127
235 H K S G L P E E D W K A R L K A R G I P F S . HIV-2cb1
235 S K S G L S E E E V R R R L T A R G L L N M A D K K E T R . SIVmac
224 Q T G K R . SYK51

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**Figure 6.** Amino acid sequence alignment of HIV/SIV Nef alleles used in this study. The mutated SF2 Nef residues are boxed; G2, P76/79 (PxxP), R109, D127, F195.

#### **4. Cell lysates and fractionation (I-IV)**

To prepare whole cell lysates, cells were washed with ice-cold standard phosphate-buffered saline (PBS) and lysed in *in vitro* kinase assay (IVKA) lysis buffer (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM ethyleneglycol-O,O'-bis-[2-aminoethyl]-N,N,N',N'-tetraacetic acid (EGTA), 1,5 mM MgCl<sub>2</sub>, containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 mM sodium orthovanadate). For fractionation into cytosolic and particulate fractions (comm. II), cells were washed with PBS, suspended into hypotonic buffer (50 mM Tris pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 1 mM EGTA) for 10 min on ice and homogenized with a Dounce homogenizer, and supernatant was cleared by centrifugation. The particulate fraction was washed with hypotonic buffer and resuspended in IVKA lysis buffer. After lysis, the extract was centrifuged and the solubilized particulate fraction collected.

#### **5. Pervanadate treatment (I)**

Pervanadate was freshly made for every experiment by incubating 100 µl of 100 mM sodium orthovanadate with 9 µl of 30% H<sub>2</sub>O<sub>2</sub> in a final volume of 200 µl for 15 min at room temperature and was kept on ice until use. Pervanadate was added to the transfected cells at a final concentration of 250 µM for 15 min, after which the cells were placed on ice, washed with cold PBS, and lysed.

#### **6. Membrane flotation by ultracentrifugation (II, III)**

Cells were washed with PBS and lysed with isolation buffer (150 mM NaCl<sub>2</sub>, 5 mM EDTA, 25 or 50 mM Tris-HCl pH 7.4, 1% Triton X-100) on ice. Samples were adjusted to 40% Optiprep (LifeTechnologies), loaded into centrifuge tubes, and overlaid with a middle layer of 28% Optiprep and a top layer of isolation buffer. After centrifugation (3 h, 35 000 rpm, 4°C), eight 500-µl fractions were collected and subjected to immunoprecipitation followed by IVKA. To detect the raft marker GM1 (in comm II), an aliquot of each fraction was transferred to polyvinylidene difluoride membranes by slot blotting and probed with horseradish peroxidase-conjugated cholera toxin. In communication III, fraction 2 representing the raft-containing fraction, and fraction 8, representing the soluble fraction were analyzed by western blotting. The quality of flotation was addressed by analyzing the localization of transferrin receptor (excluded from rafts) and LAT (incorporated in rafts).



## **7. Cholera toxin clustering in Jtag cells (III)**

At 48 h posttransfection, Jurkat Tag cells were incubated with 25 µg of Alexa 555-conjugated cholera toxin (CTx)/ml in 0.1% bovine serum albumin–PBS for 30 min at 4°C. Cross-linking was performed by incubating cells with anti-CTx antibody at a 1:200 dilution for 30 min at 4°C followed by 10 min at 37°C. Cells were seeded on poly-L-lysine-coated coverslips and fixed for 10 min with 3% paraformaldehyde–PBS. Cells were permeabilized with 0.1% Triton X (1 min, RT) and stained with the anti-tag antibodies, followed by Alexa 660-conjugated secondary antibody. Samples were analyzed with a confocal laser scanning microscope (Zeiss, LSM 510) using a 100x oil immersion objective lens.

## **8. Immunoprecipitation and *in vitro* kinase assay (IVKA) (I-IV)**

Lysates were precipitated with anti-PAK (comm. I and II) and anti-Nef (comm. II-IV) antibodies by using protein A sepharose beads (Sigma-Aldrich). For IVKA, after washing immunoprecipitates with IVKA lysis buffer and kinase assay buffer (50 mM HEPES pH 7.4 and 5 mM MgCl<sub>2</sub>), 32[P]γATP (2.5 µCi; Amersham Biosciences) was added to the immunoprecipitates followed by a 30-minute incubation at 37°C. When indicated, bacterially produced GST-substrate peptide fusion protein was added to the IVKA reaction mixture. For the tyrosine phosphorylation studies in communication I, radiolabeled NAK was eluted from anti-PAK immunoprecipitates after IVKA by incubation for 1 h at 37°C in PBS with 0.1% SDS. The eluate was diluted 10-fold with PBS and cleared with protein G-Sepharose before splitting the sample to three identical fractions subsequently re-immunoprecipitated with either empty beads (negative control), anti-phosphotyrosine antibodies (PY20), or anti-PAK antibodies. The beads were washed with PBS and boiled in Laemmli sample buffer, followed by 8-12% SDS-polyacrylamide gel electrophoresis (PAGE) and detection by autoradiography. In communication III, radioactive signals were normalized against the amount of isolated Nef in the western blot, and Nefwt.GFP signals were arbitrarily set to 100 percent. Statistical significance was determined using student's T-test.

## **9. Tyrosine dephosphorylation using PTP-1B (I)**

Protein tyrosine phosphatase 1B (PTP-1B) was expressed in bacteria as a GST fusion protein and purified from lysed cells by using glutathione Sepharose beads (Amersham Biosciences). PAK2 was immunoprecipitated from cell lysates, and beads were washed in IVKA lysis buffer and equilibrated in dephosphorylation buffer (25 mM Tris pH 7.6, 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01% Nonidet P-40, 10 µg/ml aprotinin, 1 mM sodium orthovanadate and 1 mM

PMSF. The immunoprecipitates were split into two samples and incubated with or without GST-PTP-1B (10 µg/ml) for 30 min at 35°C. After incubation, beads were washed in IVKA buffer and subjected to IVKA.

#### **10. *In vivo* labeling and phosphoamino acid analysis (I)**

Transfected cells were washed with phosphate-free medium (minimum essential medium without sodium phosphate) supplemented with 20 mM ultraglutamine-1 (BioWhittaker) and then starved in this medium for 1 hour. [<sup>32</sup>P]orthophosphate (10 mCi/ml; Amersham Biosciences) was added to achieve a concentration of 0.5 mCi/ml, and cells were labeled for 3 hours. After pervanadate treatment, cell were washed with ice-cold PBS and lysed in IVKA lysis buffer. Lysates were cleared by centrifugation (10 min, 16 000 x g) and immunoprecipitated with anti-myc antibodies. After washing with IVKA lysis buffer, the immunoprecipitates were boiled in Laemmli sample buffer and separated by 8% SDS-PAGE gel run. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and detected by autoradiography. After exposure, the labeled band corresponding to PAK2 was excised. The membrane was boiled in 6 M HCl at 110°C for 1 h. Hydrolyzed amino acids were processed as described earlier by Kamps *et al.* (Kamps and Sefton, 1989), mixed with unlabeled phosphoamino acid standards, and separated on cellulose thin-layer 20 cm x 20 cm chromatography plates (Sigma) by electrophoresis for 30 min at 2 000 V in pH 1.9 buffer (formic acid [88%]/ acetic acid/water ratio 50:156:1,794) in the first dimension and then for 20 min at 1 600 V in pH 3.5 buffer (acetic acid/pyridine/water ratio 10:1:189) for the second dimension. Plates were stained with ninhydrin to visualize the unlabeled standards; labeled amino acids were detected by autoradiography.

#### **11. Western blotting (I-IV)**

For western blotting, immunocomplexes or total protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes for subsequent immunoblotting with specific antibodies. Biotinylated secondary antibodies and streptavidin-horseradish peroxidase conjugates were used followed by enhanced chemiluminescence (ECL).

## 12. Preparation of viruses and infectivity assay (IV)

SF2 Nef wild type and the mutated variants were cloned into the pNLblue vector containing the full length proviral DNA of HIV-1 NL4-3 (Fackler *et al.*, 2006) and confirmed by sequencing. Viruses were generated by transfecting proviral HIV plasmids into 293T cells via the calcium phosphate method. Approximately 48 h after transfection, culture supernatants were harvested, cleared by centrifugation (10 min, 400 x g), and filtrated through 0.45 $\mu$ m-pore-size filter. Particles were pelleted through ultracentrifugation (90 min, 100 000 x g at 4°C) and resuspended in PBS. The CA protein concentration of concentrated stocks was determined by p24CA enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody from hybridoma cell line 183 (clone H12-5C) (Konvalinka *et al.*, 1995). The antibody for coating ELISA plates was used at a concentration of 50 ng per well. Wells were blocked with 5% milk in PBS, and appropriate dilutions of concentrated virus supernatants were added. Antigen was detected by incubation with polyclonal rabbit antiserum against HIV-1 CA, followed by horseradish peroxidase-conjugated antiserum against rabbit immunoglobulin G. Enzyme activity was detected with tetramethylbenzidine as substrate, and quantitative analysis was derived from the absorbance counts with known amounts of purified HIV-1 CA protein as standard. The relative infectivity of virus particles was determined by CA ELISA and a standardized 96-well TZM blue cell assay. Infections were carried out in triplicate with 0.5 ng CA input virus. Thirty-six hours after infection, cells were fixed with cold methanol:acetone (50:50) and stained for  $\beta$ -galactosidase. The number of blue cells was determined by microscopy.

## RESULTS

### 1. Mechanisms of PAK2 activation and the role of activation in Nef-PAK2 association (I, II)

#### 1.1. Activation of PAK2 by Cdc42 and tyrosine kinases synergize

Autophosphorylation of serine and threonine residues is the key event in maintaining the catalytically active state of PAKs. Activation is most commonly achieved by p21-GTPases Cdc42 and Rac1, but many other intracellular stimuli can also result in activation of PAK kinases, as discussed in the chapter *Regulation of PAK activity*. Activation of PAKs by p21-GTPases was previously noted to be accompanied by a shift in electrophoretic mobility of PAK in SDS-polyacrylamide gels towards forms of higher molecular weight (Bokoch *et al.*, 1998; King *et al.*, 2000). Because the results obtained earlier in our laboratory implied that HIV-1 Nef was selectively associated with the low-abundance, high-molecular-weight species of PAK2 (Renkema *et al.*, 1999; Renkema *et al.*, 2001), the mechanisms that would generate the highly active form of PAK2 became a relevant topic of more detailed studies. To investigate the possible role of tyrosine kinase signaling pathways in p21-GTPase-mediated PAK activation, three members of the SFKs - Lck, Fyn and Hck - were overexpressed in 293T cells with PAK2 and with or without the presence of a dominant active variant of Cdc42 (Cdc42V12) (comm. I, fig. 1A). Overexpressed Src tyrosine kinases alone had hardly any effect on PAK activity was hardly detectable, and they had no effect on the mobility of PAK2. However, when tyrosine kinases were expressed together with Cdc42V12, PAK autokinase activity was strongly increased. To study the role of endogenous tyrosine kinases in PAK2 activation, the cells were treated with pervanadate (PV), which is a potent tyrosine kinase inhibitor. Similarly to cells transfected only with Src kinases, pervanadate alone had little effect on PAK2 activity. When cells were cotransfected with Cdc42V12, however, pervanadate treatment resulted in a significant increase of PAK2 activity, and this activity could be inhibited in a dose-dependent manner by pretreating cells with 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1), a pharmacological Src kinase inhibitor (comm. I, fig. 1B and 1C). The order of events leading to PAK2 activation was tested by experiments in which PAK2 was immunoprecipitated from pervanadate-treated but not Cdc42V12-cotransfected cells using GTP $\gamma$ S-loaded recombinant Cdc42 to activate PAK2 *in vitro*. No difference in PAK2 activity was found between pervanadate-treated or control cells, suggesting that the activation of PAK2 by p21-GTPases primes it for further activation by Src kinases, but not vice versa (comm. I, fig. 1D).

## 1.2. Tyrosine phosphorylation of PAK2

The prominent phosphorylation of PAK2 by the synergistic effect of Cdc42V12 and Src kinases raised a question whether the effect was due to direct tyrosine phosphorylation of PAK2. Probing PAK2 immunoprecipitates from differentially transfected and treated cells with anti-phosphotyrosine antibodies did not result in specific signals (data not shown), suggesting that the bulk of PAK2 protein did not become tyrosine phosphorylated upon treatment. Taking into account the very low amount of higher-migrating, superactivated PAK2 species, immunoblotting was not considered a practical approach for identifying phosphorylation sites. Therefore, we used a more sensitive dual immunoprecipitation method, where radiolabeled PAK2 was eluted from beads and reprecipitated with either anti-phosphotyrosine or anti-myc antibodies. In this assay, neither Cdc42V12 nor pervanadate stimulation could increase PAK2 phosphorylation to the extent that would be detectable with phosphotyrosine antibody. However, from the pervanadate-treated cells with cotransfected Cdc42V12 yielded an abundant signal of phosphorylated PAK2 could be precipitated with anti-pTyr antibody (comm. I, fig. 2).

Human PAK2 contains eleven tyrosine residues. To further map the exact sites of phosphorylation, a mutational approach was used. To this end, the protein was first chopped into two parts by caspase cleavage between residues D212 and G213 (Rudel and Bokoch, 1997); re-immunoprecipitating of these fragments revealed that only the N-terminus was tyrosine phosphorylated. Thus, the tyrosine-to-phenylalanine mutations were restricted to three N-terminal tyrosine residues Y130, Y139 and Y194. By comparing anti-phosphotyrosine and anti-myc immunoprecipitations we noticed that the Y130F mutation had the most prominent effect on tyrosine phosphorylation by almost totally abolishing the signal (comm. I, fig. 2). Also, the Y194F mutation decreased the relative precipitation efficiency of the anti-pTyr antibody as compared to anti-PAK2 precipitation, making the Y194 residue an additional putative phosphoacceptor site. The Y139F mutation had less dramatic effect on phosphorylation, and as expected the triple mutant Y130/139/194F completely abolished the anti-pTyr binding. The subsequent phosphoamino acid analysis from cells transfected with Cdc42V12, treated with pervanadate and labeled *in vivo* with [<sup>32</sup>P]orthophosphate confirmed the loss of Tyr phosphorylation by the triple Y130/139/194F mutant (comm. I, fig. 3A). When similarly stimulated cells were radiolabeled *in vitro* in an IVKA reaction, the result of amino acid analysis was similar to that obtained with the triple mutant, indicating that the tyrosine phosphorylation does not take place during IVKA reaction by a coprecipitating kinase but within cells. These results implicated that the Cdc42V12-activated PAK2 was the target of direct phosphorylation and the most important residue of the PAK2 regulatory domain was Y130.

The role of Y130 as a target site of Src-mediated phosphorylation was further characterized by comparing the synergistic effect of Cdc42 and pervanadate in PAK2 wild type, PAK2-Y130F and PAK2-Y130/139/194F activation (comm. I, fig. 4). Unlike the strong potentiating effect of pervanadate on PAK2 wild type activity, pervanadate had little effect on the activity of PAK2-Y130F or the triple mutant, suggesting that the particular tyrosine residue is not only important as a site of phosphorylation, but it is also a critical residue in Src-mediated potentiation of PAK2 kinase activity.

To study the functional effects of Y130 phosphorylation, the effect of *in vitro* tyrosine dephosphorylation on PAK2 activity was tested (comm. I, fig. 5). Cdc42V12-activated PAK2 was either treated or left untreated, and half of the samples were incubated with the tyrosine phosphatase PTP-1B before subjecting it to IVKA. This treatment reduced PAK2 activity, decreasing both its autophosphorylation (4.6-fold) and activity towards the substrate peptide (3.9-fold); thus, the inhibitory effect of PTP-1B was specifically resulted from removing the phosphotyrosine modification. When different tyrosine mutants (Y130F, Y139F, Y194F, Y130/139/194F) of PAK2 activated by Cdc42 and PV were treated with PTP-1B, a significant reduction of catalytic activity was observed only in PAK2s mutated for Y139F and Y194F (2.9-fold and 4.3-fold, respectively), whereas Y130F and the triple tyrosine mutant were insensitive to treatment with PTP-1B, further confirming the role of Y130 as a major phosphoacceptor site in Src-mediated PAK2 regulation. In addition, these data demonstrated that the effect of Src kinases exerted their effect via increased catalytic activity of PAK2.

### **1.3 Tyrosine phosphorylation of PAK2 requires p21-GTPase-induced conformational changes**

Activation of PAKs by p21-GTPases is associated with disrupted autoinhibitory contacts between N- and C-termini of PAK. To examine whether p21-GTPase stimulus and the subsequent conformational change of PAK would expose tyrosine residues for phosphorylation, a panel of mutated PAK2 proteins with known conformational and functional consequences was used (comm. I, fig. 6). Mutations on H82/85L in the PAK2 PBD/CRIB domain resulting in a p21-GTPase-binding-deficient PAK2 failed to become tyrosine phosphorylated by PV treatment, even when Cdc42V12 was cotransfected. The PAK2-L106F mutant, mimicking the p21-GTPase-stimulus and resulting in open conformation and kinase activity of PAK, was also used; it was readily precipitated by anti-pTyr antibody after pervanadate treatment and did not require prior p21-GTPase stimulation. This result was also confirmed using another similar PAK2 mutant (PAK2-D125R), which yielded similar results (data not shown). To further confirm the important role of PAK2 conformation in tyrosine phosphorylation, another constitutively active PAK2,

namely PAK2-T402E, was used. This mutant is weakly responsive to p21 stimulation, but it possesses kinase activity due to the acidic residue replacement at position 402. Despite its catalytic activity, PAK2-T402E failed to become tyrosine phosphorylated upon PV treatment, even after cotransfection of Cdc42V12. Altogether, results obtained with the PAK2 mutants suggest that the conformational changes associated with p21-GTPase stimulus are required for subsequent tyrosine phosphorylation and superactivation of PAK2.

#### **1.4 Association of Nef with PAK2 is dependent on the molecular mechanism of kinase activation**

Kinase activity of PAK2 can be elicited by GTPase stimulation, mutation of the key acidic residues in the PAK regulatory domain, or by mimicking the phosphorylation event by acidic replacement of the key phosphorylation residue T402. GTPases as well as mutations of the regulatory domain yield an active kinase due to the opening of the conformation, whereas mutation T402E results in increased kinase activity without the associated conformational change. Thus, these mutants represent two structurally different approaches to mimicking PAK2 activation and serve as tools to study the effect of conformational change of PAK2 on Nef association.

To test the ability of differently activated PAK2 to associate with Nef, 293T cells were transfected with PAK2 (wild type or constitutively active mutants D125R and T402E) and laboratory-adapted HIV-1 NL4-3 strain Nef either with or without Cdc42V12 (comm. II, fig. 1). As expected, PAK2 wild type associated with Nef only when further stimulation was provided by Cdc42V12, indicating that NL4-3 Nef is a weak activator of PAK2. In contrast, when the constitutively active PAK2-D125R was used, it efficiently associated with Nef without the requirement of prior activation with the GTPase. Surprisingly, however, the autoactive PAK2-T402E did not associate with Nef both in the absence and presence of Cdc42V12, suggesting that kinase activity *per se* is not sufficient for association. Rather, the inhibitory contacts of the PAK2 N- and C-termini maintain a conformation that does not allow the formation of the Nef-PAK2 complex.

#### **1.5 Association and activation of PAK2 with HIV/SIV Nef alleles**

Because NL4-3 Nef proved poor in its ability to associate with PAK2 in the absence of kinase activation by Cdc42V12 or an appropriate mutation in PAK2, we hypothesized that NL4-3 was unable to activate PAK2. Previous studies have, however, shown that certain Nef alleles, such as HIV-1 SF2, can stimulate PAK2 activity (Arora *et al.*, 2000), suggesting that Nefs may differ in their ability to activate PAK2. Thus, we studied the capacity for PAK2 association with a panel of

six HIV-1, HIV-2, and SIV Nefs, which have all been previously characterized for their functionality and ability to associate with Cdc42V12-activated PAK2 (Kirchhoff *et al.*, 2004). Of these alleles, SIVmac239 and HIV-1 O-type alleles 8161 and 13127 were the most prominent activators of PAK2 and associated efficiently with the kinase (comm. II, fig. 2). An SIV Nef, SYK51, was as unable to stimulate or associate with the kinase as NL4-3 Nef. One HIV-2 allele, Nefcbl, was found particularly interesting, due to its strong ability to activate PAK2. Despite its obvious capacity to activate PAK2, it did not associate with PAK2 in any appreciable level. However, when PAK2 was activated with Cdc42V12, a strong signal of NAK activity could be coprecipitated with HIV-2 Nefcbl. These data suggest that activation and association with PAK2 are two separate functions of Nef. The failure of some Nefs to stimulate PAK2 activity despite the general ability of Nefs to associate with preactivated PAK2 indicates that, contrary to GTPases, PAK2 activation by Nef is not a direct consequence of binding.

#### **1.6. Cdc42 in Nef-PAK2 association**

The CRIB domain of PAK is important for its role in p21-GTPase association and the subsequent activation of PAK2 kinase, and mutations introduced to this domain abolish GTPase-induced PAK2 activity (Zhao *et al.*, 1998). Previously, Renkema *et al.* showed that substitution of two critical histidines with leucines in the CRIB domain could inhibit the association of Nef and PAK2 (Renkema *et al.*, 2001). However, it is not clear whether the dependence of NAK complex on a functional CRIB domain is due to lack of p21-GTPase-mediated activation of PAK2 or a direct consequence of lost PAK2/Cdc42-interaction. Using PAK2 proteins that were mutated within the CRIB domain (HH, H82/85L; ISP, I74N/S75P/P77A) in a cotransfection assay, we showed that both CRIB mutants could not associate with Nef (comm. II, fig. 5A), supporting the idea that the presence of p21-GTPase was required for NAK complex formation. To further test this idea, we combined our CRIB mutants with the autoactive PAK2 mutants D125R and E115K that can associate with Nef even without Cdc42. However, even when CRIB mutations (HH or ISP) were combined with conformationally active PAK2 variants to yield PAK2-E115K-HH/ISP and PAK2-D125R-HH/ISP, these mutants did not associate with Nef indicating (comm. II, fig. 5B), that presence of the p21-GTPase was required for NAK association.

However, because the loss of Nef association with active CRIB-mutated PAK2 variants could reflect the mutants' inability to localize to membranes, we studied the intracellular localization of PAK2-E115K-HH/ISP and PAK2-D125R-HH/ISP variants by transfecting them into 293T cells (comm. II, fig. 5C). Subsequently, cells were fractionated and the localization of these constructs was followed by western blotting. Interestingly, when cells transfected with these constructs were divided into



cytosolic and membraneous fractions, the E115K-HH and D125R-HH mutants were present on the membraneous fraction in amounts at least equal to, if not greater than, the p21-binding single mutants PAK2-E115K and PAK2-D125R. This result strengthened the previous findings on the indispensability of Cdc42 in NAK complex formation and led us to think that the role of p21-GTPases may physically stabilize the Nef-PAK2 association.

### **1.7. Kinase activity of PAK2 in regulation of the Nef-PAK2 complex**

The only means to verify the association of Nef with an active fraction of PAK2 has thus far been the sensitive *in vitro* kinase activity. However, the finding of p21-GTPase as an integral part of NAK complex formation led us to hypothesize that the interaction of p21-GTPase with PAK2 may take place during the activation step, with quick dissociation after kinase activity has been induced. We tested this idea by cotransfecting kinase-deficient PAK2 (K278R) with an increasing amount of p21-GTPase Cdc42V12 into 293T cells and followed the strength of interaction by western blotting (comm. II, fig. 6). Indeed, in the presence of equal amounts of PAK2-K278R and Cdc42, we could detect a visible PAK2 coprecipitation signal by Nef, suggesting that the catalytic inactivity of PAK2 stabilizes the NAK complex. In support of this view, the NAK complex was not detectable when a similar precipitation/immunoblotting approach was used for PAK2 wild type.

## **2. Localization and composition of the Nef-PAK2 complex (II, III)**

### **2.1. Intracellular localization of NAK complex**

Nef localizes partly to the cellular membrane compartment due to the myristoyl residue that is attached to the second amino acid, Gly2. The GTPases Cdc42 and Rac1 also have the CAAX motif (C is Cys, A is any aliphatic amino acid, and X is the carboxyl terminal residue) that targets them to the membrane. Once activated by Cdc42, PAK2 has been shown to localize to the ER (Roig *et al.*, 2000), but most of the PAK2 is inactive during the growing phase. However, it is not clearly established whether PAK2 is activated before localizing to the membrane or membrane localization is a prerequisite of kinase activation. Therefore, we studied the cellular compartment in which the Nef-PAK2 association would take place by transfecting 293T cells with both wild type and differentially mutated PAK2 constructs in the presence or absence of Cdc42V12 and compared the relative abundance of both PAK2 and Nef protein, as well as PAK2 and NAK activity in cytosolic and particulate (membrane) fractions (comm. II, fig. 3). In all samples, western blotting revealed equal amounts of Nef protein in both fractions, whereas PAK2 protein was mainly cytosolic. However, when immunoprecipitations and

subsequent *in vitro* kinase assays were performed, PAK2 activity was concentrated on the particulate fraction, with the exception of the PAK2-T402E mutant, whose activity remained cytosolic. Immunoprecipitations of these samples using an anti-Nef antibody detected NAK activity in the particulate fraction alone, but no NAK activity from either fraction was associated with PAK2-T402E. However, if PAK2-T402E was targeted to the membrane fraction via the CAAX-box attached to its C-terminus, this protein was capable of associating with Nef (data not shown). Thus, these data suggest that membrane-association is required for forming the NAK complex.

Lipid rafts, also referred to as detergent-resistant microdomains (DRMs), are by definition resistant to the 1% Triton-X treatment routinely used in our lysate preparation method. Because Nef was previously reported to localize to rafts (Wang *et al.*, 2000), we wanted to study whether the partitioning of NAK activity to the particulate fraction is due to its localization into rafts. To this end, the lysates from cells cotransfected with a constitutively active PAK2-E115K and NL4-3 Nef were subjected to flotation analysis by ultracentrifugation (comm. II, fig. 4). Successful flotation of rafts was followed by the presence of the raft marker ganglioside GM1. The flotation results revealed that PAK2 protein did not localize to any fraction in particular but was equally distributed throughout the fractions, whereas PAK2 activity was slightly more pronounced in the GM1-containing fraction. Nef protein was equally distributed between the cytosolic and raft-containing fractions. NAK activity was strongly concentrated on the raft-containing fraction indicating that NAK activity is more specifically localized than PAK2 activity. Remembering the association of Nef with only a small, highly active subpopulation of PAK2, this result is not surprising but rather expected. The specificity of the Nef-PAK2 interaction in rafts was further underlined by the lack of association of these proteins despite their presence in cytosolic fractions.

## **2.2. Nef-mediated recruitment of GEFs to membrane microdomains**

### **2.2.1. Ultracentrifugation of membrane microdomains**

In literature, GEFs Vav,  $\beta$ -PIX, and DOCK2/ELMO1 have been implicated as physical or functional mediators during NAK complex formation (Brown *et al.*, 1999; Fackler *et al.*, 1999; Janardhan *et al.*, 2004). The previously described presence of the NAK complex on DRMs would suggest a similar localization for the GEF that mediates the Nef-PAK2 association, making DRMs a target site for searching and validating the role of GEFs in NAK complex formation (Giese *et al.*, 2006; Krautkramer *et al.*, 2004; Pulkkinen *et al.*, 2004). To study the possible involvement of Vav,  $\beta$ -PIX, and DOCK2/ELMO1 in NAK complex formation the presence of each candidate GEF in DRMs was first analyzed by membrane

microdomain floatation studies (comm. III, fig. 1). Each GEF was cotransfected into JTag cells with either GFP or with GFP fused to either wild type Nef (Nef.GFP) or SH3-binding deficient mutant Nef-AxxA (AxxA.GFP). Membrane-residing protein LAT and soluble transferrin receptor (Tfr) were also cotransfected and used as markers for correct separation of DRM and cytosolic fractions in ultracentrifugation. Separation of the raft-containing detergent-resistant membranous fraction and the cytosolic fraction by ultracentrifugation and study of the proteins in these fractions by western blotting showed that both Nef-WT protein and the AxxA-mutant localized equally to DRM-containing fraction. Of the GEFs studied, however, both components of the DOCK2/ELMO1-complex were totally absent from the DRMs. In concert with these results, the absence of DOCK2/ELMO1 from DRMs was further confirmed by coexpression of both units of this bipartite GEF. In addition, the distribution of DOCK2/ELMO1 did not change by cotransfection of Nef, thus excluding its role in NAK complex formation.

Nef had no effect on the localization of ectopically expressed  $\beta$ -PIX, but unlike DOCK2 and ELMO1,  $\beta$ -PIX was present in DRMs regardless of the presence or absence of Nef. Endogenous  $\beta$ -PIX was more prominently present in the DRM fraction, but unlike its transfected counterpart its localization did not change in the presence of Nef.

Without Nef, transfection of Vav1 into JTag cells resulted in its total absence from the DRM fraction. However, when either Nef-wt or Nef-AxxA was cotransfected with Vav1, localization of Vav1 to the DRMs was clearly detectable with immunoblotting, suggesting the recruitment of Vav1 to DRMs by Nef. Similar results were obtained by studying the localization pattern of endogenous Vav1. However, when fractions were studied for Vav2 or active, phosphorylated Vav1, a small fraction of these proteins was detectable in DRMs in cells transfected with GFP alone. These fractions remained unaltered by expression of Nef.GFP and its derivatives. Altogether these results were compatible with the possible involvement of  $\beta$ -PIX and/or Vav1 in Nef-PAK2 association but did not support DOCK1/ELMO2 as a candidate GEF in Nef-PAK2 complex formation.

### **2.2.2. Confocal microscopy of membrane microdomains**

To confirm the results obtained by ultracentrifugation and western blotting approach, the plasma membrane microdomains of transfected JTag cells were visualized by confocal microscopy (comm. III, fig. 2). The fluorescently labeled cholera toxin subunit (CTx), which exclusively binds to the ganglioside GM1, was used to detect DRMs. In concert with the fractionation results, the diffuse cytoplasmic localization of Vav1 in the presence of GFP was changed towards more membranous localization in the presence of Nef-WT or Nef-AxxA. Both Nef

constructs distributed to the punctate structure on the plasma membranes and perinuclear membranes. The presence of the latter structures in only some Z-planal sections of cells suggest that these structures may represent endosomal membranes (Haller *et al.*, 2006; Madrid *et al.*, 2005; Thoulouze *et al.*, 2006). The colocalization of Nef with CTx in the punctate membrane structures identifies these spots as raft-containing membrane microdomains and implies that Nef is involved in the recruitment of Vav1 to these sites on cellular membranes.

Coexpression of  $\beta$ -PIX with GFP or Nef.GFP did not have an appreciable effect on the localization of either protein. The  $\beta$ -PIX was mostly found in the cytoplasm with occasional accumulation in the CTx-positive clusters, and this distribution remained unaltered by Nef. However, occasional colocalization of Nef and  $\beta$ -PIX was found in the CTx-containing microdomains, but that remained less than that of Vav1 and Nef.

The two components of bipartite GEF DOCK2/ELMO1 were diffusely localized in the cytoplasm without significant enrichment at the membrane microdomains. The localization also remained cytosolic irrespective of coexpression of GFP or Nef.GFP. Altogether, the similarity of these results to the ultracentrifugation analysis further strengthened the possible role of GEFs Vav1 and/or  $\beta$ -PIX in Nef-mediated signaling on membrane microdomains.

### **2.3. GEFs in NAK complex formation**

To further address the role of the three GEFs in the association of Nef with PAK, we developed a system to reduce the protein levels by RNAi in JTag cells. First, the maximal range of PAK activity inhibition was estimated by targeting the RNAi probe against the kinase. Even though PAK2 is typically considered the Nef-associated kinase, PAK1 has also been referred to as a possible NAK (Fackler *et al.*, 2000; Nguyen *et al.*, 2006) and was therefore included in the RNAi study.

#### **2.3.1. Knock-down of PAKs**

The RNAi oligos targeted against PAK1 or PAK2 were both efficient (knockdown of PAK1 88% +/- 15%; PAK2 94% +/-10%) and specific in knocking down the protein levels of their respective target kinases (comm. III, fig 3A). When Nef-associated PAK2 kinase activity was studied by anti-Nef precipitation and subsequent *in vitro* kinase assay from JTag cells, the control or PAK1-targeted RNAi probes did not diminish Nef-associated kinase activity, whereas treatment with PAK2-specific RNAi oligo reduced the activity to 70 % +/-19%, when NAK signals obtained from IVKA experiments were quantified by phosphoimager (comm. III, fig. 3C). As expected, the AxxA Nef mutant did not associate with PAK

kinase to any detectable amount (comm. III, fig. 3B) (Manninen *et al.*, 1998). These results confirmed that PAK2 is the principal PAK contributing to Nef-associated kinase activity. Despite the efficient knock-down of PAK2 at the protein level, PAK2 protein is still present in cells treated with RNAi to the extent that it can be efficiently recruited by Nef; thus, this complex accounts for the residual activity in IVKA, most likely due to the very high sensitivity of the IVKA method.

### **2.3.2. Knock-down of GEFs**

After determining the degree of inhibition of Nef-associated kinase activity achieved by knocking down PAKs, the effect of knocking down the previously studied GEFs on Nef-PAK2 complex was studied in JTag cells. All the GEF-targeted RNAi probes resulted in a marked reduction on the expression of their corresponding target proteins, as quantified by western blotting (comm. III, fig. 4A and 4C) (Vav1, 86% +/- 15%; Vav2, 76% +/- 10%; DOCK2, 97% +/- 5%; ELMO1, 85% +/- 9%; and  $\beta$ -PIX, 86% +/- 10%). Vav1 RNAi significantly reduced NAK activity, whereas RNAi targeted against Vav2 or ELMO1 had similar but much less pronounced effects. No alterations on NAK activity were found as a consequence of DOCK2 or  $\beta$ -PIX knock-down. Therefore, the RNAi analyses implied a role for Vav1 in the formation of the Nef-PAK complex but did not support the involvement of  $\beta$ -PIX. Together with the membrane microdomain analysis, these data suggested that of the three GEFs previously found to associate with Nef, Vav1 had the most important role in the formation of the Nef-PAK2 complex.

## **2.4. GEF-deficient cell lines and $\beta$ -PIX-binding-deficient PAK2 in NAK activity studies**

To strengthen the results from JTag cell line on the contribution of Vav1, as well as the dispensable role of  $\beta$ -PIX and DOCK2 on Nef-associated PAK2 activity, we used either cell lines deficient for expression of Vav1 and DOCK2 or a transfection approach using PIX binding-deficient PAK2.

First, a Vav1-deficient Jurkat cell line (Cao *et al.*, 2002), JVav, was used to complement the RNAi studies on the role of Vav1 on Nef-associated PAK2 activity. In concert with the RNAi results, the capacity of HIV-1 SF2 Nef to associate with PAK2 activity was reduced by approximately 70% in JVav cells as compared to JTag cell line (comm. III, fig. 5A). In addition, the reduced NAK activity in JVav cells was conserved for SIVmac Nef, HIV-1 NL4-3 NefT71R mutant, and patient-derived HIV-1 Nef variant RP4-11 (comm. III, fig 5B). To confirm the importance of Vav1 for Nef-PAK2 association, JVav cells were transfected with Vav1 (comm. III, fig. 5C). This cotransfection increased NAK activity almost to the level of NAK activity in JTag cells. Because Nef can associate also with Vav2 (Fackler *et al.*,

1999), the possible contribution of Vav2 to the residual Nef-PAK2 association in JVav cells was examined using a Vav2 knock-down approach. Although the knock-down efficiency of Vav2 was only partial in JVav cells, it reduced NAK activity to 54% +/- 15% for HIV-1 SF2 Nef and 41% +/- 20% for SIVmac239 Nef (comm. III, fig. 5D). Altogether, these results support the idea that Vav1 contributes to Nef-PAK2 complex formation, and suggest that Vav2 serves as a substitute GEF in the absence of Vav1.

To analyze the role of DOCK2 for NAK complex formation, a DOCK2<sup>-/-</sup> BE $\alpha$ 16-3 T-cell hybridoma cell line was used (Sanui *et al.*, 2003). No significant difference in association of PAK activity either with HIV-1 or SIV Nef was observed using JTag and BE $\alpha$ 16-3 cells (comm. III, fig. 6A), suggesting the dispensability of DOCK2 in NAK association. Notably however, a 50% reduction in Nef-associated kinase activity reduction was seen in BE $\alpha$ 16-3 cells when Vav1 was knocked-down by RNAi (comm. III, fig. 6B). Because no cell line with  $\beta$ -PIX<sup>-/-</sup> genetic background was available, we used PAK2 mutated for its  $\beta$ -PIX binding site (P185G/R186A) (Manser *et al.*, 1998) in our experiments (comm. III, fig. 6C). Again, no difference in either HIV-1 or SIV Nef-associated PAK2 activity was observed in the presence of either PAK2-wt or  $\beta$ -PIX binding-deficient PAK2 mutant. Combined with the results of the RNAi experiments and the DRM recruitment studies, these data supported the idea that Vav1 was the GEF with the most prominent functional role for Nef-PAK2 complex formation.

## **2.5. The role of Nef residue F195 in Nef-PAK2 association and Vav1 recruitment**

The highly conserved proline-rich SH3-binding domain (PxxP) of Nef has previously been reported to be critical for association with NAK (Khan *et al.*, 1998; Manninen *et al.*, 1998; Wiskerchen and Cheng-Mayer, 1996), which fits with the finding of that SH3-containing Vav1 mediates Nef-PAK2 association. However, this is not in concert with our data on PxxP-independent recruitment of Nef into the DRMs. Recent literature has described a hydrophobic protein interaction surface including a particular residue F195 of SF2Nef, that is specifically involved in association of Nef and PAK2 (Agopian *et al.*, 2006; Raney *et al.*, 2005). To test the importance of residue F195 for the formation of the Nef-PAK2 complex, two mutations F195A and F195I were introduced to the SF2Nef sequence. Both mutations efficiently impaired association with PAK2 by binding worse than the previously described association-deficient controls Nef-G2A or Nef-AxxA (comm. III, fig. 7). Using these mutants in raft flotation experiments and confocal microscopy (comm. III, fig. 8), we confirmed that the lack of kinase association was not a consequence of exclusion of F195 mutants from membrane microdomains or their distinct localization pattern in T lymphocytes. In contrast, both of the mutated

F195 variants failed to recruit Vav1 into the membrane microdomain-containing fraction in ultracentrifugation experiments. Respectively, no relocalization of Vav1 from the cytosol to the CTx-rich sites on cell membranes could be detected in the presence of Nef-F195 mutants. Thus, Nef residue F195 is a critical amino acid in Nef-PAK2 association, and its involvement in Vav1 recruitment to membrane microdomains is important for Nef-mediated signaling.

### **3. The role of putative dimer interface in Nef functions (IV)**

Mutations residing in the PDI of Nef have previously been reported to affect several Nef functions (Fackler *et al.*, 2006; Liu *et al.*, 2000; Manninen *et al.*, 1998). To study the functional role of residues R105 and D123, which may contribute to the stability of Nef dimers, we took advantage of a mutational strategy (comm. IV, fig. 1). We reasoned that reversing either of these charges by R105D or D123R mutations should inhibit PDI-mediated Nef dimerization, whereas a double mutation R105D/D123R might rescue the self-association capacity of Nef and the possible functional defects associated with failed dimerization.

#### **3.1. Enhancement of viral infectivity**

HIV-1 Nef has an established role in increasing the infectivity of the virus. Moreover, mutations within the Nef PDI have been associated with decreased virion infectivity (Fackler *et al.*, 2006; Liu *et al.*, 2000). To study whether complementary charges that stabilize the interaction surface are required for the increased viral infectivity by Nef, we produced empty and Nef-deleted ( $\Delta$ Nef) control viruses as well as viruses expressing either Nef wild type or its PDI-mutated counterparts in 293T cells and tested these viruses in viral infectivity assay (comm. IV, fig. 2). All Nef constructs and viral control proteins p55Gag and p24CA were equally expressed as analyzed by western blots from producer cells. When the infectivity of these viruses was studied in a single-round replication assay of TZM cells, viruses expressing wild type Nef were approximately 70% more infectious than  $\Delta$ Nef viruses. Introduction of R105D and D123R mutations into Nef dramatically reduced virion infectivity to the level of Nef-deleted viruses, but the viral infectivity could be fully restored when the double mutant Nef R105D/D123R was used.

#### **3.2. Demonstration of Nef dimers by coprecipitation**

Taken the central role that has been suggested for R105 and D123 residues in the self-association of Nef molecules, we investigated whether we could reconstitute association of Nef molecules by direct immunoprecipitation (comm. IV, fig. 3). To this end, we transfected 293T cells with Nef R105D, D123R or R105D/D123R

variants tagged C-terminally either with a myc-epitope or biotinylation sequence (Nef-PP). Using horseradish peroxidase-conjugated streptavidin detection for cell lysates immunoprecipitated with anti-myc antibodies, we showed that biotinylated Nef WT was efficiently pulled down by myc-tagged Nef WT. In comparison to Nef wt, all three Nef constructs with mutations at the interaction interface showed reduced coprecipitation efficiency, but none of the Nef variants was fully incapable of self-association. Despite the decreased coprecipitation of the mutant Nef pairs, our coprecipitation experiments could not confirm that a R105/D123-mediated interaction between Nef molecules was involved in Nef dimerization or that the rescued infectivity of Nef-R105D/D123R in infectivity assay was due to a rescued capacity to dimerize.

### **3.3. Association with PAK2 activity**

Association with active PAK2 is a common characteristic of HIV-1/2 and SIV Nefs (Kirchhoff *et al.*, 2004). In addition to the domains within the Nef core (Agopian *et al.*, 2006; Fackler *et al.*, 2006; Manninen *et al.*, 1998), a specific Nef residue F191 selectively disrupts Nef-PAK2 association without affecting other Nef functions (Agopian *et al.*, 2006). To test the importance of Nef's surface charge distribution for its interaction with PAK2, we transfected 293T cells with either Nef wild type or Nef-R105D, D123R or R105D/D123R mutants (comm. IV, fig. 4). In addition, Nef-F191R was used as a negative control for PAK2 association. Cell lysates were subjected to immunoprecipitation with an anti-Nef antibody; coimmunoprecipitated active PAK was labeled in *in vitro* kinase assay, run on SDS-PAGE gel and detected by autoradiography. In accordance with previously described data, wild type SF2 Nef readily associated with active PAK2 (Arora *et al.*, 2000), whereas both Nef-F191R and R105D did not associate with PAK2. In contrast, Nef-D123R was totally dispensable for association with PAK2 activity. Moreover, the doubly mutated Nef-R105D/D123R markedly, though not completely, restored the Nef-R105D-PAK2 association.

### **3.4. Membrane association**

Membrane association of Nef is mediated largely by myristoylation of its N-terminus (Yu and Felsted, 1992). We and others have previously demonstrated that the Nef-PAK2 complex specifically localizes to cellular membranes (Krautkramer *et al.*, 2004; Pulkkinen *et al.*, 2004). Therefore it was of interest to study whether the capacity of Nef mutants to associate with active PAK2 would correlate with their ability to associate with membranes. To this end, we transfected 293T cells with Nef wt or mutated Nef variants, fractionated the cells into cytosolic and particulate fractions, and followed the distribution of Nef protein by western blot analysis of



these fractions (comm. IV, fig. 5). As expected, most Nef protein was detected in the cytosolic fraction. Nef wt and Nef-D123R proteins were also robustly detectable in the particulate fraction, whereas the amount of Nef-R105D protein on the particulate fraction was reduced. However, like PAK2 activation, this defect could be rescued by a dual mutation, and Nef-R105D/D123R again fractionated like Nef wt.

### **3.5. Hck activation**

Src tyrosine kinase Hck is an important mediator of Nef effects in hematopoietic cells. Forced oligomerization and hydrophobic patch residues of Nef have previously been shown to contribute to the association and activation of Hck by Nef (Choi and Smithgall, 2004; Ye *et al.*, 2004), but more detailed characterization on the role of charged residues in Nef self-association surface is lacking. To address the importance of the charge distribution on Nef interaction surface during Hck activation, we transfected 293T cells with Hck fused to a biotinylation tag (Hck-PP) together with Nef wild type or its mutated derivatives. Hck was precipitated from cell lysates using streptavidin beads, and its phosphorylation status was detected with a specific antibody that recognizes phosphorylated S211 and Y209 residues, which are associated with release of Hck autoinhibition upon activation (comm. IV, fig. 6). As expected, Nef wt induced robust phosphorylation of Hck, whereas no Hck phosphorylation signal was detected by the negative control Nef-AxxA. When Nefs carrying charge-reversing mutations were assayed, Nef-R105D was as efficient as Nef wild type in its ability to activate Hck. In contrast, Nef-D123R was clearly less efficient in Hck phosphorylation. Again, this effect could be rescued by introducing the reciprocal D123R mutation to the PDI, resulting in wild type-like Hck activation via Nef-R105D/D123R.

## DISCUSSION

### 1. Activation of PAK2 by Src kinases and the role of activation in Nef-PAK2 association

Compared to its relatively small size, HIV-1 pathogenicity factor Nef possesses a stunning multiplicity of associating proteins and cellular functions. Structurally, the flexible arm and loop domains provide conformational flexibility that enables its adaptor function. The multitude of Nef functions and interactions has also been envisioned as a consequence of the “Nef-cycle”: the conformational differences between closed, membrane-bound Nef, and open, cytosolic protein (Arold and Baur, 2001).

Many Nef interactions with endogenous host cell proteins have clearly defined functional consequences, but the *in vivo* effects of Nef-PAK2 association have remained elusive. The ability of Nef to selectively associate with the highly active PAK2 species led us to elucidate the intracellular mechanisms leading to increased kinetic activity of PAK2. In this study, a novel mode of PAK2 superactivation by the sequential action of p21-GTPases and SFKs was described. In resemblance to the conformation-dependent potentiation of PAK2 activity described in communication I, we also found that the association of PAK2 with Nef required structural alterations in the kinase. NL4-3 Nef associated with PAK2 only after p21-GTPase-stimulated PAK2 activation or when an ‘open-conformation’ mutant PAK2-D125R was used. The lack of Nef association with PAK2-T402E, even after p21 stimulation, suggested that here the opened conformation of PAK2 molecule was critical for NAK activity. The capacity to associate with PAK2 activity is a conserved function of various Nef alleles (Kirchhoff *et al.*, 2004). However, Nefs varied greatly in their ability to themselves stimulate PAK2 activity, as shown by the results in communication II. Our findings suggest that activation of PAK2 by Nef and Nef’s association with PAK2 are mechanistically separate functions, although the capacity of Nef to associate with active PAK2 is used as an indicator of its capacity to activate PAK2. The capability of the HIV-2cbl Nef allele to activate PAK2 without associating with PAK implied that Nef may also modulate protein activities that affect PAK activity, e.g. signaling pathways upstream of p21-GTPases, without requirement of direct association. The fact that several described Nef-interacting proteins are GEFs, i.e. activators of p21-GTPases, further underpins this view.

Despite the obviously important role of catalytic PAK2 activity for its interaction with Nef, the strong destabilizing effect of kinase activity was also a limiting factor for Nef-PAK2 association. The cotransfection studies using kinase-dead PAK2 together with Cdc42 and Nef showed that decreased kinase activity resulted in a more stable complex that could be visualized even with coimmunoprecipitation and

subsequent western blotting, which has not been possible for NAK complex possessing wild type PAK2 activity. The highly transient nature of the complex formed by PAK2 wild type, p21-GTPase, and Nef may thus explain the difficulties in its detection.

## 2. Composition of the Nef-PAK2 complex

Because both Nef and active PAK2 have previously been detected in the cell membranes, it was not surprising that the Nef-PAK2 complex localized to rafts, also defined as DRMs. At the time of our publication (Pulkkinen *et al.*, 2004), the association of NAK with DRM was also shown by another group, but in addition to the presence of NAK complex in DRMs, their work suggested Nef as a factor to recruit PAK to membranes with subsequent activation (Krautkramer *et al.*, 2004). Our experiments did not confirm the contribution of Nef in recruitment of PAK2 to membranes, since active PAK2 was equally present in the raft fraction both in the presence and absence of Nef. Nef was also detected in fractions other than the raft-specific one, yet NAK activity was clearly most abundant in the raft-containing fraction, which may indicate that other proteins, such as GTPases, are limiting factors that target the active NAK complex specifically to rafts (del Pozo *et al.*, 2004).

By using a number of doubly mutated PAK2 variants (PAK2-E115K-CRIB/ISP and PAK2-D125R-CRIB/ISP) with open conformation and deficiency in p21GTPase-binding, we showed that localization of PAK2 to the particulate fraction was not dependent on the p21-GTPase activity. A very recent study by Parrini *et al.* has described a three-stage activation cascade of PAK, where the authors suggest that the first 'semi-activation state' of PAK is achieved by membrane targeting induced by interaction with Nck/Grb2 adaptor proteins (Parrini *et al.*, 2009) required to expose PAK for further activation by GTPases and by other associating proteins. In concert with our findings, this model supports the role of GTPases as PAK-stimulating, but not membrane-recruiting factor *per se*. Despite the ability of CRIB-mutated PAK2-E115K and PAK2-D125R variants to traffic to membranes, they could not associate with Nef. This implies that p21-binding has more significant effect on NAK complex formation than solely the activation of PAK2. In support of this view, we could indeed confirm the role of Cdc42 in stabilizing the Nef-PAK2 interaction by experiments using kinase-dead PAK2 and Nef in the immunoprecipitation studies in communication II.

The presence of an SH3-containing protein as a mediator of Nef-PAK2 interaction has often been discussed, because the association of the two is abolished by mutating the SH3-binding domain in Nef (Manninen *et al.*, 1998). The detailed composition of the Nef-PAK2 complex has however remained elusive due to its

transient nature and the difficulties with direct detection of associating proteins. In communication III, we used a combination of approaches to overcome the limitations of each method, and characterized Vav1 as a GEF binding partner associated with the NAK complex. Indeed, Vav1 has previously been suggested as a possible SH3-containing mediator protein required for Nef-PAK2 association (Fackler *et al.*, 1999; Quaranta *et al.*, 2003; Vilhardt *et al.*, 2002). Unexpectedly, however, we found that the proline-rich domain in Nef was not required for recruitment of Vav1 to the NAK complex, but the interaction was dependent on Nef F195 residue, recently depicted as critical for the Nef-PAK2 association (Agopian *et al.*, 2006). This finding leaves the PxxP-binding component of the PAK2-Nef complex uncharacterized, leaving the possibility of yet another, unidentified protein component open. Moreover, the SH3-PxxP interaction by Nef and Vav1 may stabilize the complex or Vav GEF activity rather than recruit protein components, which could explain the irrelevant role of the proline-rich Nef domain in recruitment studies.

Although both DOCK2/ELMO1 and  $\beta$ -PIX have been reported to associate with Nef, the experimental strategies used here did not confirm their role in the Nef-PAK2 complex. The absence of DOCK2/ELMO1 in DRMs or CTx clusters in cells expressing Nef argued against a role of this GEF in Nef-PAK2 association. Furthermore, the use of DOCK2-deficient BE $\alpha$ 16-3 cells verified that DOCK2 expression was dispensable for NAK activity. These results are not in conflict with the original findings by Janardhan *et al.* on association of Nef with DOCK2/ELMO1 (Janardhan *et al.*, 2004), but they support the idea of association of Nef-PAK2 in a separate complex independently of DOCK2/ELMO1.

In a similar manner, RNAi knockdown of  $\beta$ -PIX or the use of PIX-binding deficient PAK2 mutants did not significantly affect the association of Nef with PAK2 activity, suggesting dispensability of  $\beta$ -PIX for Nef-PAK2 association. Although this GEF has previously been shown as a physical component of the Nef-PAK2 complex (Vincent *et al.*, 2005) and as a substrate of PAK2 (Brown *et al.*, 1999), it is possible that  $\beta$ -PIX associates with Nef-PAK2 complex as a more peripheral component that does not contribute to PAK2 activity or acts as a downstream effector of the NAK complex (Simmons *et al.*, 2005).

The SFK family members Fyn and Lck, studied in communication I, are both central kinases that mediate the downstream signaling initiated by TcR stimulus and play an important role in T cell development and activation of mature T cells (Salmond *et al.*, 2009). The activation of SFKs, in particular Lck and Fyn, is one of the earliest events in intracellular signaling after TcR ligation, followed by the formation of the proximal signaling complex that initiates the downstream signaling cascade. Several components of the proximal signaling complex, such as ZAP-70, Nck, and Vav1 have been described as mediators of PAK activity and/or localization (Bokoch *et al.*,

1996; Fackler *et al.*, 1999; Ku *et al.*, 2001; Lu *et al.*, 1997). Redistribution of Lck by Nef, as well as Nef-induced interference with cell spreading and actin remodeling upon TcR stimulus have been associated with impaired formation of the immunological synapse (Haller *et al.*, 2007; Thoulouze *et al.*, 2006). In addition, a recent report shows that Nef can alter cell motility in a PAK-dependent manner (Stolp *et al.*, 2009). The identity of the protein that Nef initially targets to result in Nef-PAK2 association is unclear. However, these current data from T cells suggests that NAK activity may provide the virus a means to modulate host cell migratory properties. The third SFK studied in communication I, Hck, is different from Lck and Fyn, because it is most abundantly expressed in phagocytic cells. There, it is involved in phagocytosis, cell movement and actin remodeling (Guet *et al.*, 2008). Interestingly, the capacity of Nef to activate Hck by direct SH3-mediated binding also places Nef as a potential upstream activator of PAK2 via Src kinases. Although no direct evidence that Hck, PAK2 and Nef are present in the same multiprotein complex exists, this does not exclude the possibility that these proteins and activation events could take place within the same signalosome. Accumulating knowledge on the multitude on proteins participating in the TcR signaling has made it evident that these signaling cascades are not linear, starting from the TcR ligation and ending at transcriptional regulation at the nucleus. Instead, complex feedback and feed forward regulation is present at each step.

### **3. The PDI and Nef functions**

Initially, evidence on Nef oligomerization was provided by X-ray crystallography (Arold *et al.*, 2000; Lee *et al.*, 1996) and NMR spectroscopy studies (Grzesiek *et al.*, 1997) suggesting self-association of Nef core domains. These interactions were mediated by conserved Nef core residues and further stabilized by the complementary charges of R105 and D123. The amino acids of this hydrophobic patch on the Nef core domain have been later reported to have roles in several Nef functions, including downregulation of CD4 and MHC I, viral infectivity increase, as well as NAK activity (Liu *et al.*, 2000; Manninen *et al.*, 1998). In communication IV, we reciprocally mutated the stabilizing residues R105 and D123 in the PDI of Nef. The fact that single complementary mutations of these residues led to aberration of several critical Nef functions that could be rescued by the reciprocal charge reversion at both sites supports the idea that the PDI can mediate self-association of Nef and that the functional consequences of single mutations are due to failed self-association of Nef.

Our initial hypothesis was that disrupting Nef dimerization mediated by reciprocal charges of R105 and D123 would result in a severely defective or full loss-of-function phenotype in Nef. Respectively, functional phenotype could be restored when a double mutation R0109D/D123R was introduced, indicating recovery by

restored dimerization capacity. This explanation, however, seems oversimplified. The results obtained in immunoprecipitation experiments where mutations introduced to the PDI had only a minute inhibitory effect on the Nef self-association capacity also support this view. In addition to structural predictions and NMR techniques, previous studies have used immunoblotting and Nef-fused inducible dimerization domain approaches to demonstrate oligomerization of Nef. Our results are not in conflict with these findings, as precipitation of Nef wt molecules also occurred in our experiments; rather, our studies suggest that the reciprocal charges by R105 and D123 are not a determining factor to putative dimerization of Nef. However, they clearly contribute to Nef functions, possibly by coordinating Nef conformation. Another contributing mechanism to the readily detectable self-association by Nef-R105D and D123R mutants may be that, in addition to dimerization, Nef molecules form higher oligomers in a manner that is independent of PDI residues. Formation of such large multimers has been suggested, implicating that Nef oligomers may have further tendency to aggregate (Fujii *et al.*, 1996).

Altogether, results obtained in communication IV suggest that the charges provided by Nef residues R105 and D123 coordinate its various functions. This may rather be due to the important contribution provided by these charges to the interactions formed between Nef and endogenous signaling proteins instead of a strong functional role of Nef self-assembly *in vivo*. However, these data do not exclude the possibility of Nef dimer formation, especially in conditions where other cellular interaction partners are sparse. Our results provide novel *in vivo* insight into the self-association of Nef, whose role in Nef functions has previously only been based on *in vitro* data.

## CONCLUSIONS AND PERSPECTIVES

HIV-1 Nef affects a variety of signaling cascades and other crucial activities in an infected cell by interacting with endogenous proteins. Given the role of Nef as a critical pathogenicity factor of HIV-1, characterizing Nef interactions and their functional consequences *in vivo* is necessary to identify potential target molecules for therapeutical intervention.

In this work, we have analyzed the association of Nef with a serine/threonine kinase PAK2. We identified PAK2 as a convergence point for Src kinase- and p21-GTPase-mediated signaling. The central role of these cascades in T cell signaling and regulation of actin remodeling suggests that the superactivation of PAK2 by the two convergent signals may contribute to the adhesion and migratory properties of cells. In addition to p21-GTPases, conformational changes associated with PAK2 activation were also found necessary for its association with Nef. Interestingly however, we also showed that Nef could activate PAK2 without their direct association within the same protein complex. This implies that Nef may modulate PAK2 activity via currently uncharacterized mechanisms.

The association of Nef and PAK2 is dependent on the PxxP domain of Nef. As PAK2 lacks SH3 domain, this implies the presence of an additional SH3 domain-containing protein. In this work, SH3 domain-containing Vav1 was found to be the GEF component of the Nef-PAK2 complex. Unexpectedly, however, Vav1 recruitment to the Nef-PAK2 'signalosome' was not mediated by the Nef PxxP domain, although Nef association with Vav1 has previously been mapped to Vav1 SH3. This leaves the identification of yet another complex component open for future study.

Our experiments to elucidate the functional role of the Nef PDI indicated that several Nef functions were defective upon the single mutation of the complementary charges R105 and D123, but these functions could be rescued by the double mutation that caused a reciprocal charge change. The importance of the complementary charges for Nef functions suggested that the PDI could also be envisioned as a potential target site for Nef-inhibitory drugs.

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## **12. ORIGINAL COMMUNICATIONS**

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## Cdc42/Rac1-Mediated Activation Primes PAK2 for Superactivation by Tyrosine Phosphorylation

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**The involvement of p21-activated kinases (PAKs) in important cellular processes such as regulation of the actin skeleton morphology, transduction of signals controlling gene expression, and execution of programmed cell death has directed attention to the regulation of the activity of these kinases. Here we report that activation of PAK2 by p21 GTPases can be strongly potentiated by cellular tyrosine kinases. PAK2 became tyrosine phosphorylated in its N-terminal regulatory domain, where Y130 was identified as the major phosphoacceptor site. Tyrosine phosphorylation-mediated superactivation of PAK2 could be induced by overexpression of different Src kinases or by inhibiting cellular tyrosine phosphatases with pervanadate and could be blocked by the Src kinase inhibitor PP1 or by mutating the Y130 residue. Analysis of PAK2 mutants activated by amino acid changes in the autoinhibitory domain or the catalytic domain indicated that GTPase-induced conformational changes, rather than catalytic activation per se, rendered PAK2 a target for tyrosine phosphorylation. Thus, PAK activation represents a potentially important point of convergence of tyrosine kinase- and p21 GTPase-dependent signaling pathways.**

The mammalian family of *Saccharomyces cerevisiae* Ste20-like kinases consists of six members known as p21-activated kinases 1 to 6 (PAK1 to -6). Phylogenetic analysis clusters the more recently identified PAK4, PAK5, and PAK6 as a distinct subfamily (PAK-II) separate from that of PAK1, PAK2, and PAK3 (PAK-I subfamily) (10, 15). The majority of the published data on PAK kinases still concern the members of the PAK-I subfamily, which serve important cellular functions involved in regulation of the actin cytoskeleton morphology, transduction of signals controlling gene expression, and execution of programmed cell death (for reviews, see references 3 and 11).

All PAKs contain a conserved serine/threonine kinase domain that comprises the C-terminal half and an N-terminally located p21-binding domain (PBD; also called the Cdc42/Rac-1 interactive binding [CRIB] domain) (24). PAK-I members also contain several characterized motifs for the interaction with signaling molecules (3, 11). These motifs include an N-terminal proline-rich region (PxxP motif), which can target PAKs to the plasma membrane and mediate interactions with transmembrane growth factor receptors via binding to the second SH3 domain of the adapter protein Nck (4, 22, 38), and another SH3-ligand motif that mediates binding to the alpha-PAK-interacting exchange factor ( $\alpha$ -PIX) and  $\beta$ -PIX (also called COOL-1 and COOL-2, respectively) (5, 25). The cellular substrates of PAK that mediate the downstream effect of these kinases are still incompletely characterized but have been reported to include Bad and myosin heavy and light chains, as well as myosin regulatory light chain kinase, LIM kinase, Raf-1, p47<sup>phox</sup>, and MEK-1 (11).

The emerging roles of PAK kinases in regulation of multiple

fundamental cellular processes have directed significant attention into understanding how the activity of these kinases is controlled. Biochemical and structural studies have revealed autoinhibition of the C-terminal catalytic domain by the N-terminal domain as the key mechanism in regulation of PAKs. Several layers of inhibition, involving dimerization and occupation of the catalytic cleft by contacts between the N- and C-terminal domains, keep the catalytic activity of PAK kinases in check (21; reviewed in reference 14). Autoinhibition of PAK1 has recently been shown to occur in *trans*, meaning that the inhibitory domain of one PAK1 molecule interacts with the kinase domain of another PAK1 molecule (30). Association of GTP-bound forms of Cdc42 or Rac1 with the PAK PBD/CRIB domain induces conformational changes in the N-terminal domain that no longer support its autoinhibitory function. Thus, the activity of PAKs can be induced by a variety of extracellular signals that control the cellular factors that regulate the GTP association state of Cdc42 or Rac1. As alternative or complementary mechanisms for p21-mediated PAK activation, binding of certain sphingolipids, such as sphingosine, and caspase-catalyzed proteolytic cleavage between the N- and C-terminal domains (in the case of PAK2) have also been shown to be able to overcome PAK autoinhibition (7, 19).

Autophosphorylation of serine and threonine residues is another key event in establishing and maintaining a catalytically active state of PAKs. Of the several serine residues that become phosphorylated upon PAK activation, Ser141 (numbered according to PAK2) has been reported to be particularly important (8). Similarly to that of many other kinases, phosphorylation of the activation loop in the kinase domain of PAK plays a dominant role the activation process (12, 37). The site of this phosphorylation is Thr402, which is the only threonine residue that becomes autophosphorylated in PAK (12, 23). An acidic T402E substitution of this residue is sufficient to render PAK catalytically active autonomously of the N-terminal reg-

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ulatory region. It has been suggested that the earliest stages of PAK activation in cells may involve phosphorylation of this threonine residue by 3-phosphoinositide-dependent kinase-1 instead of by PAK autophosphorylation (17).

The catalytic activity of PAKs is accompanied by a shift in the electrophoretic mobility in sodium dodecyl sulfate (SDS)-polyacrylamide gels towards forms of higher apparent molecular weight (7, 17). An observation that focused our interest on PAK regulation was that pathogenicity factor Nef of human immunodeficiency virus type 1 specifically associated with a high-molecular-weight species of PAK2 which, despite its low relative abundance, accounted for most of the cellular PAK2 activity (31, 32). Our present studies, aimed at characterization of the mechanisms that govern generation of this highly active PAK2 species, have revealed a novel and potentially important feature of PAK regulation. Here we show that induction of PAK2 activity via p21 GTPases can be strongly potentiated by concurrent stimulation of cellular tyrosine kinase activity. Overexpression of various Src kinases or treatment of cells with the tyrosine phosphatase inhibitor pervanadate had little effect on PAK2 activity on their own but enhanced the ability of Cdc42 or Rac1 to stimulate PAK2 activity. This potentiation was mediated by phosphorylation of Y130 in the N-terminal regulatory domain. Together, the biochemical and genetic data from these studies support a model in which p21 GTPase binding-induced conformational changes render PAK2 a substrate for subsequent Src kinase-mediated tyrosine phosphorylation, leading to a robust enhancement of the catalytic activity of PAK2.

#### MATERIALS AND METHODS

**Cell culture, transient transfections, and pervanadate treatment.** 293T human embryonic kidney cells (American Type Culture Collection) were maintained under standard culture conditions. Transfections were performed using Lipofectamine transfection reagent (Gibco BRL) according to the manufacturer's instructions. Cells were used for experiments 48 h after transfection. Pervanadate was freshly made for every experiment by incubating 100  $\mu$ l of 100 mM sodium orthovanadate with 9  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub> in a final volume of 200  $\mu$ l for 15 min at room temperature and was kept on ice until use. Pervanadate was added to the transfected cell in a final concentration of 250  $\mu$ M for 15 min, after which the cells were placed on ice, washed with ice cold phosphate-buffered saline, and lysed.

**Plasmids and construction of PAK2 mutants.** The PAK2-encoding insert was cloned in a pEBB eukaryotic expression vector with either a MycHis-HA (hemagglutinin) multiepitope (ME) tag (32) or a MycHis (MH) tag. Since the ME tag used in our previous studies contains a HA tag with potential tyrosine phosphorylation sites, the MH-tagged proteins were used in most experiments. Unless otherwise indicated in the respective figure legend, the PAK2 constructs were MH tagged. All PAK2 mutants were made by overlap PCR, using a specific mutant primer pair and common outer primers containing restriction sites used for cloning. The expression plasmid for dominant active (V12) Cdc42V12 was kindly provided by B. Mayer.

As a substrate for the PAK2-kinase assays, we cloned three tandem copies of the 7-amino-acid peptide of the Rous sarcoma virus nucleocapsid (KKRKSGL) in frame with glutathione S-transferase (GST) into the bacterial expression vector pGEX-4T-1 (Amersham Biosciences). This peptide was shown by Tuazon et al. to contain the consensus substrate sequence for PAK2 (36). In contrast to myelin basic protein, this substrate did not promote p21-independent activation of PAK2 (data not shown).

**Immunoprecipitations and in vitro kinase assays.** Immunoprecipitations using anti-Myc (9E10) antibodies and subsequent in vitro kinase assays (IVKA) were performed as described previously (32). When indicated, bacterially produced GST-substrate peptide fusion protein (100  $\mu$ g/ml) was added to the IVKA reaction mixture. To visualize incorporation of <sup>32</sup>P into the substrate peptide, a fraction of the kinase assay was analyzed using SDS-12% polyacrylamide gel electrophoresis (PAGE).

**Tyrosine dephosphorylation using PTP-1B.** Protein tyrosine phosphatase 1B (PTP-1B) was expressed in bacteria as a GST fusion protein and purified from the lysed cells by using glutathione Sepharose beads (Amersham Biosciences). PAK2 was immunoprecipitated from cell extracts, and beads were washed in IVKA lysis buffer and equilibrated in dephosphorylation buffer (25 mM Tris [pH 7.6], 10% [vol/vol] glycerol, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01% Nonidet P-40, including protease inhibitors). The beads were split in two identical samples and incubated with or without GST-PTP-1B (10  $\mu$ g/ml) for 30 min at 35°C. After the incubation, the beads were washed in IVKA buffer and subjected to IVKA.

**In vivo labeling and phosphoamino acid analysis.** Transfected cells were washed with phosphate-free medium (minimum essential medium without sodium phosphate) (Sigma) supplemented with 20 mM Ultraglutamine-1 (Biowhitaker) and starved in this medium for 1 h. [<sup>32</sup>P]orthophosphate (10 mCi/ml; Amersham Biosciences) was added to achieve a concentration of 0.5 mCi/ml, and cells were labeled for 3 h. After pervanadate treatment, the cells were washed with ice-cold phosphate-buffered saline and lysed in IVKA lysis buffer. Lysates were cleared by centrifugation (10 min, 16,000  $\times$  g) and immunoprecipitated with anti-Myc antibodies. After extensive washing with IVKA lysis buffer, the immunoprecipitates were boiled in SDS-PAGE sample buffer and separated by SDS-8% PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad), and after exposure to X-ray film, the labeled PAK2 band was excised. The membrane piece was boiled in 6 M HCl at 110°C for 1 h. Hydrolyzed amino acids were processed as described previously (16), mixed with unlabeled phosphoamino acid standards, and separated on cellulose thin-layer chromatography plates (20 cm long by 20 cm wide; Merck) by electrophoresis, using a HTLE-7000 system for 30 min at 2,000 V in pH 1.9 buffer (formic acid [88%]/acetic acid/water ratio, 50:156:1,794) in the first dimension and for 20 min at 1,600 V in pH 3.5 buffer (acetic acid/pyridine/water ratio, 10:1:189) for the second dimension. Plates were stained with ninhydrin to visualize the unlabeled standards; labeled amino acids were visualized by autoradiography.

## RESULTS

**Synergistic regulation of PAK2 activity by Cdc42 and Src family tyrosine kinases.** As analyzed by IVKA and SDS-gel electrophoresis, activation of PAK2 by cotransfection of a dominant active variant of Cdc42 (Cdc42V12) into 293T cells resulted in increased PAK2 autokinase activity and the appearance of slower-migrating, differentially phosphorylated PAK2 species (Fig. 1A, two leftmost lanes). In agreement with the previous data of Renkema et al. (32), the Cdc42-induced high-molecular-weight PAK2 species exhibited a very high specific activity, as this band became intensely labeled in the kinase reaction whereas only the PAK2 species migrating below was abundant enough to be detected by immunoblotting. Similar results were obtained when a dominant active variant of Rac1 was used instead of Cdc42 (not shown).

To study possible involvement of tyrosine kinase signaling pathways in modulation of p21 GTPase-mediated PAK activation, we transfected cells with various combinations of PAK2, Cdc42V12, and different Src family tyrosine kinases, including Fyn, Hck, and Lck. As shown in Fig. 1A, overexpression of these kinases alone had only a marginal effect on PAK2 kinase activity and no effect on the mobility of PAK2 protein. Strikingly, however, overexpression of both Cdc42V12 and a Src kinase resulted in a significant potentiation of PAK2 autokinase activity.

To study whether endogenous tyrosine kinases could have a similar effect on PAK2 activation, we treated the Cdc42V12-transfected cells with pervanadate, a potent inhibitor of tyrosine phosphatases. This also resulted in a strong increase in PAK2 kinase activity (Fig. 1B), which was equally evident when assayed as autophosphorylation (top panel) or as kinase activity towards a peptide containing three copies of a PAK2-sub-

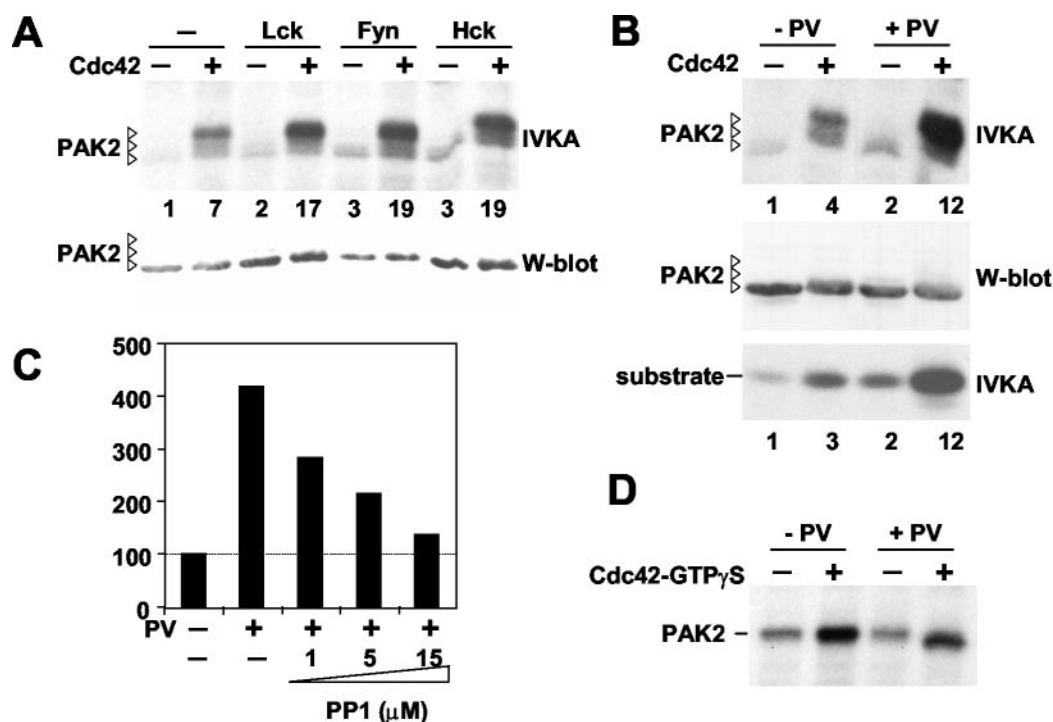


FIG. 1. Potentiation of Cdc42-induced catalytic activation by overexpression of Src family kinases or by inhibition of cellular tyrosine kinase activity. (A) PAK2 was transfected into 293T cells in various combinations with or without different Src kinases and Cdc42V12, as indicated. PAK2 proteins were precipitated with an anti-Myc antibody and subjected to autophosphorylation in the presence of [ $^{32}$ P]ATP. Half of the radiolabeled reaction mixtures were analyzed by anti-Myc Western blotting (lower panel), and the other half were analyzed by direct autoradiography of the gel (upper panel). PAK2 autophosphorylation in each lane was quantified by phosphorimager analysis of the gel and is indicated as a severalfold increase relative to the value obtained without cotransfection of Cdc42V12 and a Src kinase. (B) PAK2 expression, autokinase activity, and kinase activity towards the substrate peptide, with (+) or without (-) cotransfection of Cdc42V12, was examined as for panel A. Instead of Src kinase overexpression, however, tyrosine phosphorylation was induced by addition of pervanadate (PV) 15 min before lysis of the cells as indicated. PAK2 autophosphorylation and kinase activity in each lane was quantified by phosphorimager analysis of the gel and is indicated as a severalfold increase relative to the value obtained without activation. (C) Graph representation of phosphorimager-quantified autophosphorylation of PAK2 from cells cotransfected with PAK2 and Cdc42V12, analyzed as for panel B. The leftmost bar is normalized to 100% and represents PAK2 autokinase activity without pervanadate treatment (-). The four other bars indicate PAK2 activity in cells treated with pervanadate alone (+) or in combination with a 45-min pretreatment with Src kinase inhibitor PP1 (1, 5, or 15  $\mu$ M). (D) The effect of pervanadate treatment of cells on PAK2 activity was examined as described above, except that PAK2 was transfected in the absence of Cdc42V12 or other activator proteins. Instead, PAK2 activity in half of the immunoprecipitated material was induced in vitro by addition of recombinant GTP $\gamma$ S-loaded Cdc42 into the IVKA reaction mixture as indicated.

strate consensus sequence (36) (bottom panel). As was observed by transfecting Src kinases, pervanadate stimulation had little effect unless the cells were cotransfected with an active p21 GTPase. Similar synergistic PAK2 activation by pervanadate was observed in COS-7 cells and in 3T3 cells transfected with either Cdc42V12 or Rac1V12 (data not shown). Pretreatment of cells before the pervanadate stimulation with PP1, a pharmacological inhibitor of Src kinases, abolished the potentiation of Cdc42-induced PAK2 activation in a dose-dependent manner (Fig. 1C). We therefore concluded that the endogenous tyrosine kinase involved in the synergistic activation of PAK2 was likely to be a Src kinase family member also.

When PAK2 was immunoprecipitated from pervanadate-treated cells that had not been transfected with Cdc42V12 and was only subsequently activated in vitro by using GTP $\gamma$ S-loaded recombinant Cdc42, no difference in the extent of activation of PAK2 derived from pervanadate-treated or control cells was observed (Fig. 1D). This suggested that induction of cellular tyrosine phosphorylation could not prime PAK2 for

subsequent activation by p21 GTPases and that the reverse order of events was the case.

**PAK2 becomes directly tyrosine phosphorylated.** We next wanted to determine whether the synergy of Src kinases and p21 GTPases involved direct tyrosine phosphorylation of PAK2. Probing anti-PAK2 immunoprecipitates from differentially transfected and stimulated cells with anti-phosphotyrosine antibodies gave no specific signal, indicating that, regardless of whether Cdc42V12 was transfected or not, the bulk of PAK2 did not become tyrosine phosphorylated even upon pervanadate treatment (data not shown). However, considering that the slower-migrating, superactivated species of PAK2 was not abundant enough to be detected by anti-PAK2 immunoblotting (Fig. 1), anti-phosphotyrosine Western blotting would not be expected to be a suitable strategy for examining the phosphorylation status of this subpopulation of PAK2. Therefore, a more sensitive experimental strategy was adopted. PAK2, radiolabeled by IVKA, was eluted from the immunobeads and reprecipitated using an anti-phosphoty-



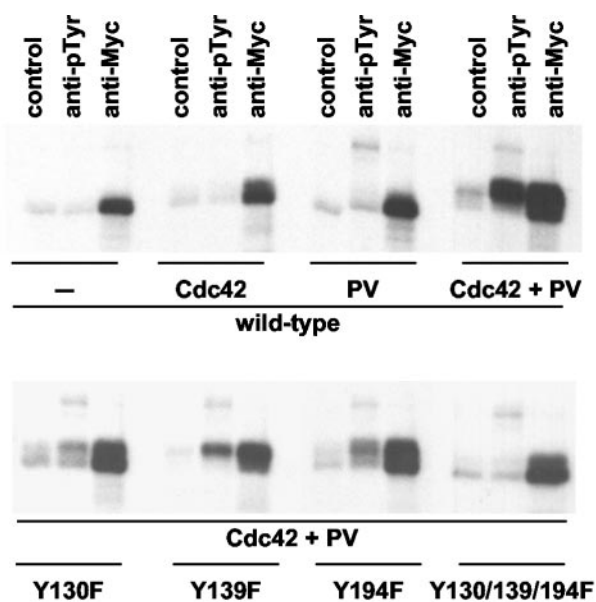


FIG. 2. P21-activated PAK2 becomes a target for Y130 phosphorylation. 293T cells were transfected with wild-type PAK2 (upper panel) or PAK2 constructs with individual point mutations or a triple mutation disrupting the three potential N-terminal tyrosine phosphorylation sites (lower panel) and were cotransfected with Cdc42V12 and treated with pervanadate (PV) as indicated. After immunoprecipitation and IVKA, the labeled PAK2 proteins were eluted and split into three identical fractions that were subsequently reimmunoprecipitated with empty beads (control), anti-phosphotyrosine antibodies (anti-pTyr), or anti-PAK antibodies (anti-Myc).

rosine antibody (PY20) or an anti-PAK (anti-Myc-tag) antibody as a positive control. As shown in the upper panel of Fig. 2, cotransfection of Cdc42V12 together with pervanadate treatment resulted in a PAK2 subpopulation that could be readily reimmunoprecipitated with the anti-pTyr antibody. By contrast, cotransfection of Cdc42V12 alone did not give rise to a PAK2 subpopulation that could be detected by the anti-pTyr antibody. Most notably, pervanadate treatment of cells not transfected with Cdc42V12 failed to induce significant tyrosine phosphorylation of PAK2. Similar results were also obtained with a different monoclonal anti-pTyr antibody (4G10; data not shown). In anti-pTyr precipitates from pervanadate-treated cells, an additional upper band was also seen. We have previously noted this protein to be phosphorylated in anti-PAK2 IVKA assays. And we believe it to be PIX or a PIX-associated protein, as its presence was dependent on an intact PIX-binding motif in PAK2 (32). Indeed, when a PAK2 mutant with a disrupted PIX-binding motif was used, this protein was not seen (data not shown). Notably, this had no effect on the ability of the anti-pTyr antibodies to reimmunoprecipitate PAK2, indicating that PIX or another PIX-associated phosphoprotein, such as G-protein-coupled receptor kinase-interacting target (GIT) or paxillin, did not help to mediate precipitation of PAK2 by the anti-pTyr antibodies.

To determine which tyrosine residues in PAK2 were the targets for phosphorylation, a mutagenesis approach was undertaken. Since human PAK2 contains 11 tyrosine residues, we first determined in which part of the protein the target tyrosine residue(s) was located by making use of the ability of PAK2 to

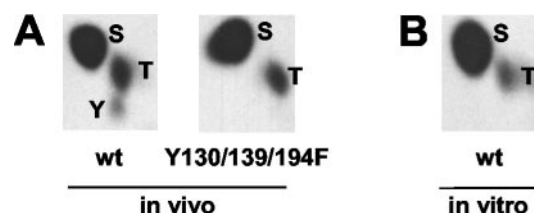


FIG. 3. Phosphoamino acid analysis of PAK2. (A) Analysis of wild-type PAK2 and PAK2-Y130/139/194F from cells that were cotransfected with Cdc42v12 and treated with pervanadate and labeled in vivo with [ $^{32}$ P]orthophosphate. (B) Analysis of wild-type PAK2 labeled in vitro by IVKA reaction with [ $^{32}$ P]ATP after immunoprecipitation from cells that were cotransfected with Cdc42v12 and treated with pervanadate.

be cleaved by caspase 3 between residues 212 and 213 (35). Reimmunoprecipitations of the caspase 3 cleavage fragments of PAK2 indicated that only the N-terminal half of PAK2 was tyrosine phosphorylated (data not shown). Therefore, we mutated the three tyrosine residues found in the N-terminal half of PAK2 into phenylalanines to create the mutants Y130F, Y139F, and Y194F and used the reimmunoprecipitation assay to test their capacity to become tyrosine phosphorylated (Fig. 2, bottom panel). Comparison of the abilities of the anti-Myc and anti-pTyr antibodies to precipitate the mutant proteins revealed that the Y130F mutation alone almost completely abolished tyrosine phosphorylation. By contrast, the Y139F could still be recognized by the anti-pTyr antibody. Also, the Y194F mutation appeared to decrease the relative ability of anti-pTyr (compared to anti-Myc) to precipitate this protein, suggesting that Y194 may be an additional phosphoacceptor site. As expected, when all three mutations were combined into the same molecule (Y130/139/194F), the anti-pTyr precipitation was completely lost.

In agreement with the reimmunoprecipitation findings, phosphoamino acid analysis of cells labeled in vivo with [ $^{32}$ P]orthophosphate revealed a phosphotyrosine signal from the wild-type PAK2-expressing cells but not the Y130/139/194F-expressing cells cotransfected with Cdc42V12 and stimulated with pervanadate (Fig. 3A). No  $^{32}$ P-labeled phosphotyrosine was detected from wild-type PAK2 immunoprecipitated from similarly activated unlabeled cells and subsequently radiolabeled in vitro by IVKA (Fig. 3B), indicating that tyrosine phosphorylation of PAK2 takes place in the cells, rather than during the IVKA assay due to a coprecipitating kinase. From these results, we concluded that Cdc42V12-activated PAK2 is susceptible to direct tyrosine phosphorylation and that Y130 in its regulatory domain is the main target residue.

**Tyrosine phosphorylation increases the intrinsic catalytic activity of PAK2.** To directly study the role of Y130 in the tyrosine phosphorylation-mediated activation of PAK2, we compared the ability of Cdc42V12 and pervanadate to synergistically activate wild-type PAK2, PAK2-Y130F, and PAK2-Y130/139/194F. As is evident in Fig. 4, unlike its effect on that of the wild type, pervanadate had little additional effect on kinase activity of Y130F and Y130/139/194F mutant proteins. Thus, we concluded that Y130 in the N-terminal regulatory domain is not only the predominant site for tyrosine phosphor-

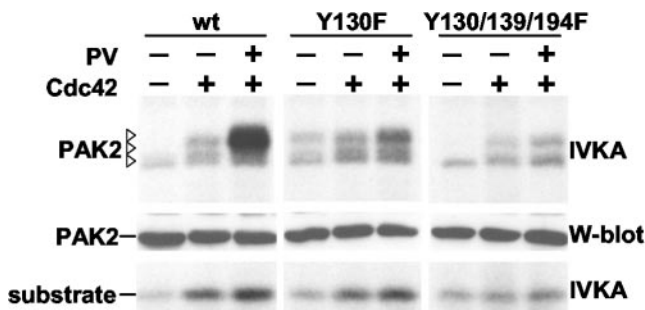


FIG. 4. PAK2-Y130 is critical for synergistic activation by Cdc42 and pervanadate treatment. 293T cells were transfected with wild-type PAK2, PAK2-Y130F, or PAK2-Y130/139/194F and were cotransfected with Cdc42V12 and treated with pervanadate (PV) as indicated. Protein expression and autokinase activity and kinase activity towards the substrate peptide was examined as for Fig. 1B.

ylation but is also the critical target residue for Src kinase-mediated potentiation of PAK2 kinase activity.

To further characterize the functional role of PAK2 Y130 phosphorylation, we tested the effect of *in vitro* tyrosine dephosphorylation on PAK2 kinase activity. To this end, Cdc42V12-pervanadate-activated PAK2 was treated with the tyrosine phosphatase PTP-1B before subjecting it to IVKA (Fig. 5). This treatment potently reduced PAK2 activity, decreasing its autophosphorylation by 4.6-fold and its activity towards the substrate peptide 3.9-fold, thus virtually abolishing the superactivating effect of pervanadate. By contrast, the activity of PAK2 induced by Cdc42V12 alone without pervanadate stimulation was not sensitive to PTP-1B, indicating that the inhibition by this treatment was indeed specifically due to removal of the phosphotyrosine modification.

When the different tyrosine mutants of PAK2 activated by Cdc42V12 plus pervanadate were treated with PTP-1B, a significant reduction in the catalytic activity of Y139F and Y194F (2.9-fold and 4.3-fold, respectively) was observed, whereas the

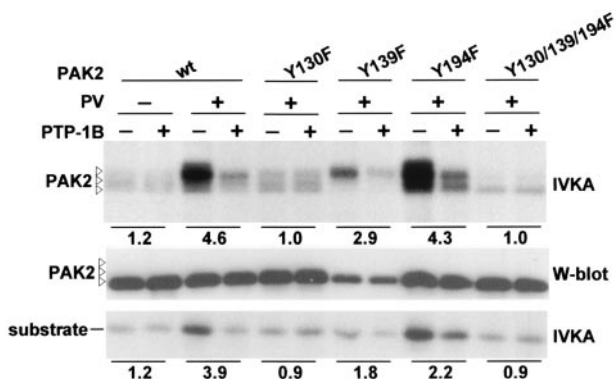


FIG. 5. Tyrosine phosphorylation increases the specific activity of PAK2. PAK2 and tyrosine mutants were immunoprecipitated from cells that were cotransfected with Cdc42V12 and treated or not treated with pervanadate (PV) as indicated. Half of the immunocomplexes were incubated with PTP-1B, as indicated, to remove phosphate from tyrosine residues before they were subjected to IVKA. Numbers indicate the severalfold decrease (compared to the untreated sample) in autophosphorylation or kinase activity towards the substrate peptide, as determined by phosphorimager analysis of the gels.

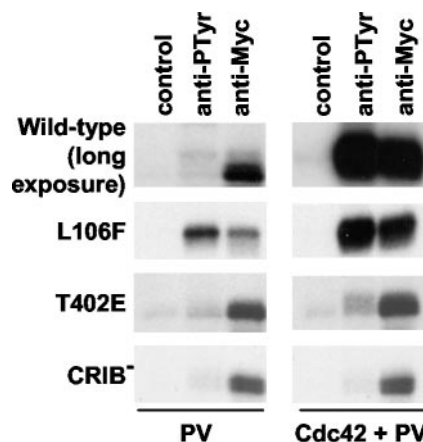


FIG. 6. PAK2 tyrosine phosphorylation is dependent on Cdc42-induced conformational changes. Different PAK2 mutants carrying the ME tag were transfected with or without cotransfection of Cdc42V12 as indicated and treated with pervanadate (PV). After primary immunoprecipitation and IVKA, samples were eluted and reimmunoprecipitated, using empty beads (control), anti-phosphotyrosine antibodies (anti-PTyr), or anti-PAK antibodies (anti-Myc). The autoradiograph showing results obtained with wild-type PAK2 was exposed six times longer than the other panels.

Y130F and the Y130/139/139F mutants were completely insensitive to PTP-1B treatment. Although tyrosine 194, in addition to the major phosphoacceptor site Y130, was also found to be phosphorylated (Fig. 2), modification of Y194 did not seem to be involved in positive regulation of PAK2 by Src kinases. In fact, the activity of PAK-Y194F in Cdc42V12 plus pervanadate-stimulated cells was slightly higher than that of wild-type PAK2. In conclusion, these data further confirm the critical role of Y130 in tyrosine kinase-mediated regulation of PAK2 activity and provide a direct demonstration that this regulation is achieved via an increased intrinsic catalytic activity of PAK2.

**Tyrosine phosphorylation is dependent on p21-induced conformational changes in PAK2.** Activation of PAKs by Cdc42 involves disruption of the autoinhibitory interactions between the N-terminal regulatory domain and the C-terminal kinase domain (14, 21, 30). Since PAK2 tyrosine phosphorylation was dependent on prior activation by Cdc42, we considered the possibility that p21 binding-induced changes in the PAK2 structure would lead to exposure of potential sites for tyrosine phosphorylation. To test this possibility, we examined a panel of previously characterized function-modifying PAK2 mutations for their effects on tyrosine phosphorylation of PAK2.

Mutations H82L and H85L, which inactivate the PBD/CRIB domain, result in a PAK2 protein (PAK2-CRIB<sup>-</sup>) that has some constitutive activity but is resistant to further activation by Cdc42V12. In agreement with our previous conclusions regarding the requirement of prior action by a p21 GTPase for PAK2 tyrosine phosphorylation, PAK2-CRIB<sup>-</sup> failed to become tyrosine phosphorylated in pervanadate-treated cells even when cotransfected with Cdc42V12 (Fig. 6). A very different picture was seen when the PAK2-L106F mutant was tested. The L106F mutation mimics the action of p21 GTPases by partially abolishing the autoinhibition exerted by the N-terminal domain and results in a PAK2 mutant which has an increased preference for an open conformation and significant

constitutive activity. Similarly to that of Cdc42-stimulated wild-type PAK2, however, the fraction of total cellular PAK2-L106F present in the shifted conformation remained too small to be readily detected by Western blotting (data not shown). Unlike wild-type PAK2, PAK2-L106F became intensely phosphorylated on tyrosine residues in pervanadate-treated cells even in the absence of Cdc42V12 (Fig. 6). Identical results were obtained when another conformationally activating mutant (PAK-D125R) was used (data not shown). To confirm that the open conformation of PAK2-L106F, rather than its increased catalytic activity per se, was important for its targeting to tyrosine phosphorylation, we tested another type of constitutively active PAK2 mutant. PAK2-T402E is catalytically active because of an acidic substitution involving the T402 autophosphorylation site in its kinase domain. This mutant is also poorly responsive to activated p21 GTPases, as indicated by only a small further increase in kinase activity in Cdc42V12-transfected cells (Fig. 6 and results not shown). Despite its constitutive activity, PAK2-T402E did not become tyrosine phosphorylated upon pervanadate treatment when transfected alone, and unlike wild-type PAK2 and PAK2-L106F, it did not become significantly tyrosine phosphorylated in pervanadate-stimulated cells even when cotransfected with Cdc42V12 (Fig. 6). Of note, despite an intact PBD/CRIB motif, PAK2-T402E resembled PAK-CRIB<sup>-</sup> in that it failed to undergo the characteristic shift in mobility indicative of an altered conformation and/or autophosphorylation status when cotransfected with Cdc42V12.

Thus, collectively these data suggest a model in which conformational changes associated with p21-mediated activation target PAK2 for subsequent tyrosine phosphorylation, which in turn leads to robust enhancement of the catalytic activity of PAK2.

## DISCUSSION

In this study, we describe a new signaling mechanism that regulates PAK kinase activity. Activation of PAK2 by p21 GTPases was found to be significantly potentiated by overexpression of Src family tyrosine kinases or treatment of cells with pervanadate, a potent inhibitor of cellular tyrosine phosphatases. This effect of pervanadate could be abolished by the presence of the Src kinase inhibitor PP1. The enhancement of PAK2 activity by Src kinases involved tyrosine phosphorylation of PAK2 itself, which occurred in the N-terminal regulatory domain, where Y130 was identified as the major phosphoacceptor site. The positive effect of tyrosine phosphorylation on the catalytic activity of PAK2 was direct, since specific removal of these phosphates using the tyrosine phosphatase PTP-1B also abolished the increased activity of PAK2.

Perhaps the most interesting aspect of this phosphotyrosine-based regulation was its strict dependence on prior activation of PAK2 by p21 GTPases. Wild-type PAK2 could only become phosphorylated on tyrosine residues when an active Cdc42 or Rac1 was cotransfected, whereas a mutant form of PAK2 unable to interact with p21 GTPases (PAK2-CRIB<sup>-</sup>) did not undergo tyrosine phosphorylation, even when such an activator was provided. The major acceptor site for tyrosine phosphorylation in PAK2 was mapped to residue Y130. The crystal structure of PAK1 (21) shows that this residue would be hid-

den when PAK2 is in its autoinhibited conformation. Binding of a GTP-loaded p21 GTPase results in an open, catalytically active conformation of PAK2 in which Y130 would become exposed and available for phosphorylation by Src kinases. In direct support of such conformational regulation of PAK2 tyrosine phosphorylation, we observed that PAK2 mutants that were constitutively active (because of amino acid changes that oppose the closed conformation of PAK2 by interfering with the autoinhibitory contacts between its N- and C-terminal domains [L106F and D125R]) could be tyrosine phosphorylated independently of p21 GTPases. By contrast, this was not the case with PAK2-T402E, which is constitutively active because of a mutation in its catalytic apparatus. In addition to such activation-associated changes in PAK structure, it is of course possible that other concurrent effects of Cdc42/Rac1 binding on PAK2, such as membrane recruitment (33) and breakdown of dimers (30), also contribute to the observed p21 GTPase-dependent Src kinase action on PAK2.

While the concept of Cdc42/Rac-dependent PAK tyrosine phosphorylation leading to subsequent catalytic superactivation is novel, previous studies have implicated certain non-Src tyrosine kinases in PAK regulation. Traugh and colleagues have reported that overexpression of c-Abl can result in phosphorylation of a significant fraction of cellular PAK2 (34). However, in contrast to the effect of Src kinases reported here, phosphorylation by c-Abl was not dependent on cotransfection of an active p21 GTPase, and instead of potentiating PAK2 activity, it caused a sixfold decrease in the specific activity of PAK2. By contrast, tyrosine phosphorylation by the Etk/Bmx kinase (2) or by v-ErbB (28) has been reported to be associated with an increased activity of PAK1. Although the transforming action of v-ErbB itself appeared to be Rho dependent (6, 28), in neither of these cases did PAK tyrosine phosphorylation require activated Cdc42 or Rac, which led the authors of the latter study to suggest that tyrosine phosphorylation represents a p21 GTPase-independent mechanism of PAK activation (28). The sites of tyrosine phosphorylation of PAK by c-Abl, v-ErbB, or Etk/Bmx were not mapped in any of these studies, but it would seem logical to assume that these sites are different from that of the PAK2 Y130 (or those of the corresponding residues in PAK1 and PAK3).

The coordinated regulation of PAK2 by p21 GTPases and tyrosine phosphorylation bears intriguing resemblance to regulation of Raf-1, another important and more extensively studied upstream regulator of cellular serine/threonine kinase cascades. Raf-1 is synergistically activated by binding to the p21 GTPase protein Ras and by tyrosine phosphorylation by Src (reviewed in reference 29). In this case also, prior action by Ras is required for the stimulatory phosphorylations to occur at Ser338 and Tyr341 of Raf-1 (27). The role of Ras in this process is to translocate Raf-1 to the membrane where these modifications take place (20, 26), but notably, a cysteine-rich domain adjacent to the Ras binding domain of Raf-1 has also been implicated in autoinhibition by a physical interaction with the catalytic region of this kinase, in direct analogy with PAK regulation (9). Moreover, similar to the multistep process of PAK2 activation described here, only a small proportion of total cellular Raf-1 becomes activated by stimuli that activate Ras (13), and therefore, despite the importance of Tyr341 phosphorylation for maximal Raf-1 activity, only a small sub-



population of Raf-1 molecules becomes tyrosine phosphorylated by Src, which may explain why this modification has not been observed in all studies (see references 1 and 18, for example). Thus, although a number of differences in PAK and Raf-1 regulation are also apparent, the complex molecular mechanisms that govern the catalytic activities of these kinases may have more in common than has been previously appreciated.

In conclusion, the p21 GTPase-dependent tyrosine phosphorylation of the Y130 residue, leading to superactivation of PAK2, represents a novel point of convergence for signal transduction pathways mediated via small G proteins and Src family tyrosine kinases. This mechanism may be important in providing additional plasticity and complexity to the regulation of cellular activities of PAK and thus allowing a cell to respond appropriately to different extracellular stimuli and could also have relevance for development of novel therapeutic strategies based on protein kinase inhibitors.

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## Nef Associates with p21-Activated Kinase 2 in a p21-GTPase-Dependent Dynamic Activation Complex within Lipid Rafts

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**We have previously reported that Nef specifically interacts with a small but highly active subpopulation of p21-activated kinase 2 (PAK2). Here we show that this is due to a transient association of Nef with a PAK2 activation complex within a detergent-insoluble membrane compartment containing the lipid raft marker GM1. The low abundance of this Nef-associated kinase (NAK) complex was found to be due to an autoregulatory mechanism. Although activation of PAK2 was required for assembly of the NAK complex, catalytic activity of PAK2 also promoted dissociation of this complex. Testing different constitutively active PAK2 mutants indicated that the conformation associated with p21-mediated activation rather than kinase activity per se was required for PAK2 to become NAK. Although association with PAK2 is one of the most conserved properties of Nef, we found that the ability to stimulate PAK2 activity differed markedly among divergent Nef alleles, suggesting that PAK2 association and activation are distinct functions of Nef. However, mutations introduced into the p21-binding domain of PAK2 revealed that p21-GTPases are involved in both of these Nef functions and, in addition to promoting PAK2 activation, also help to physically stabilize the NAK complex.**

The Nef protein is a 25- to 34-kDa accessory protein of primate immunodeficiency viruses (i.e., human/simian immunodeficiency virus [HIV/SIV]) and a major determinant of in vivo pathogenicity of these lentiviruses. Nef promotes viral replication via numerous, but incompletely understood strategies (13, 14). Nef has been found in lipid rafts (35), which are important microdomains in signal transduction. Nef modulates cellular signaling events, downregulates major histocompatibility complex class I and CD4 cell surface expression, and contributes to the infectivity of virus particles (7, 27). One potentially important cellular effector of Nef is the Nef-associated serine/threonine kinase (NAK) (23, 30), later identified as p21-activated kinase 2 (PAK2) (1, 25). The interaction between Nef and PAK2 takes place in a multiprotein complex (12) and critically depends on the stabilizing effects of other components of this complex, which remain unidentified but apparently include at least one protein that can bind to the SH3 domain-binding site of Nef (21). NAK has been implicated as an effector of Nef in inhibition of Bad-mediated apoptotic death and in increasing virus production in HIV-infected cells (19, 36).

The PAKs are mammalian homologues of yeast Ste20-like protein kinases and can be divided into two subfamilies: PAK-1, -2, and -3 (the PAK-I subfamily) and PAK-4, -5, and -6 (the PAK-II subfamily). Signaling via PAKs is involved in regulation of actin cytoskeleton, apoptosis, and malignant transformation (2, 5, 11, 16, 33, 34). Catalytic activation of PAKs

typically results from binding of the GTP-loaded Rho p21-GTPases Cdc42 or Rac1 to the Cdc42/Rac interaction/binding (CRIB) domain (also known as p21-binding domain) located in the N-terminal regulatory domain. This binding relieves its autoinhibitory contacts with the C-terminal kinase domain and is followed by phosphorylation on Thr402 (PAK2; Thr423 in PAK1), as well as other key autophosphorylation sites and kinase activation (4, 22).

In addition to a net increase in cellular GTPase activity, PAKs may also be activated by their increased recruitment toward active GTPases, for example, by Nck-mediated plasma membrane targeting of PAK1 (3, 20). On the other hand, Cdc42-independent activation mechanisms induced by lipids, such as sphingosine, have been reported for both PAK1 and PAK2 (3, 28). Sphingosine stimulation can induce a similar level of activity as do p21-GTPases, as well as a similar pattern of autophosphorylation, and is associated with translocation of PAK2 to a membrane-containing cellular fraction (28).

Among the total cellular pool of PAK2, only a small but highly active fraction associates with Nef (1, 26). Because of the selective association of HIV type 1 (HIV-1) Nef with the catalytically active fraction of cellular PAK2, we have undertaken here a detailed analysis of the relationships between p21-GTPase binding, catalytic activity, subcellular localization, and association with Nef by PAK2. These results provide new insights into the role of p21-GTPases in Nef/PAK2 complex formation, as well as in the process of PAK activation itself.

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### MATERIALS AND METHODS

**Cell culture and transient transfections.** 293T human embryonic kidney fibroblast-derived cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and Glutamax. Transfections were

performed by using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested 48 h after transfection.

**Plasmid constructs.** The generation of PAK2-HH (H82/85L) and PAK2-T402E has been described earlier (26). The PAK2 mutants ISP (I74N/S75P/P77A), E115K, and D125R were made by overlap PCR with a specific mutant primer pair and common outer primers containing restriction sites for cloning. All constructs were cloned into pEBB-ME with BamHI-KpnI to generate a multi-epitope (ME)-tagged construct (26). The PAK2 double mutants with E115K or D125R mutations combined to HH or ISP mutations were made by cloning the HH- or ISP-mutated N-terminal fragment to the pEBB-PAK2-ME vector containing the respective autoinhibitory mutation. All constructs were confirmed by sequencing. The expression plasmid for dominant active Cdc42<sup>V12</sup> was kindly provided by B. Mayer. pEBB-NL4-3-Nef R71 has been described previously (29). All divergent Nef alleles were cloned into pCG plasmid, and they are described elsewhere (15), except for HIV-2 cbl and SIV SYK51. HIV-2 cbl Nef was amplified from an HIV-2-infected culture obtained through the NIH AIDS Research and Reference Reagent Program. The Nef allele SYK51 was kindly provided by Beatrice Hahn (Department of Medicine, University of Alabama at Birmingham [B. Hahn, unpublished data]).

**Cell lysates and fractionation.** To obtain whole-cell lysates, cells were washed once with cold phosphate-buffered saline (PBS) and suspended in *in vitro* kinase assay (IVKA) lysis buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml). After 10 min on ice, the extract was centrifuged (13,000 rpm, 10 min), and the supernatant was collected. For fractionation of PAK2 into cytosolic and particulate fractions, cells were washed with PBS, suspended in hypotonic buffer (50 mM Tris [pH 7.5], 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA) for 10 min on ice, and homogenized by 20 strokes with a Dounce homogenizer. After centrifugation (13,000 rpm for 10 min) the supernatant was collected. The particulate fraction was washed once with hypotonic buffer and resuspended in IVKA lysis buffer. After 10 min on ice, the extract was centrifuged at 13,000 rpm for 10 min, and the solubilized particulate fraction was collected.

**Raft fractionation by membrane flotation.** Cells were washed once with PBS and lysed with ice-cold isolation buffer (150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml). After 20 min incubation on ice, samples were cleared by centrifugation (1,000 × g, 10 min). Lysates were adjusted to 40% Optiprep (Life Technologies), pipetted into Sorvall TH-660 centrifuge tubes, overlaid with 2.5 ml of 28% Optiprep in isolation buffer and with 0.6 ml of isolation buffer, and centrifuged for 3 h at 35,000 rpm at 4°C. Eight 500-μl fractions were collected from the top and subjected to immunoprecipitations with anti-Myc or anti-Nef antisera followed by IVKA analysis. A small part of each immunoprecipitate was boiled in sample buffer, separated in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to nitrocellulose membrane for Western blot analysis. From each fraction, 50 μl was transferred to polyvinylidene difluoride membranes by slot blotting and probed with 200 ng of horseradish peroxidase-conjugated cholera toxin (CTX; Sigma)/ml to detect the raft marker GM1.

**Immunoprecipitations and IVKAs.** Immunoprecipitations with anti-Myc (9E10), anti-AU1 (Babco, Richmond, Calif.), or a monoclonal antibody (2F2; FIT Biotech, Tampere, Finland) and subsequent IVKAs with [ $\gamma$ -<sup>32</sup>P]ATP were performed as described previously (26). To visualize the autophosphorylation activity of PAK2, a fraction of the kinase assay was subjected to sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis and exposed to X-ray film.

## RESULTS

**Nef association depends on the molecular mechanism of PAK2 activation.** Mutations affecting acidic residues in the regulatory domain of PAK1 have been shown to result in a constitutively active kinase by disrupting intramolecular autoinhibitory interactions that suppress the catalytic domain (18, 38). Here, we have produced constitutively active PAK2 variants by mutating the corresponding residues in it (E115K and D125R) or, alternatively, by a strategy commonly applied to generate constitutively active protein kinases, namely, acidic replacement of a key phosphorylation site in the kinase activation loop (T402). 293T cells were transfected with wild-type or different constitutively active PAK2 variants and HIV-1

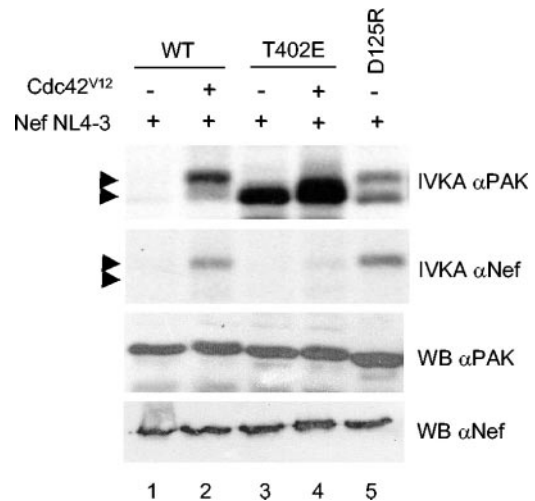


FIG. 1. Activation-dependent association of PAK2 with Nef. 293T cells were transfected with HIV-1 NL4-3 Nef, together with wild-type (WT) PAK2 or constitutively active PAK2 variants (D125R and T402E). In some transfections Cdc42<sup>V12</sup> was also included, as indicated in the figure. Cell lysates were immunoprecipitated with anti-Myc-tag antibodies ( $\alpha$ PAK; top panel) or anti-Nef antibodies ( $\alpha$ Nef; middle panel), followed by an IVKA in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. The expression of total PAK2 and Nef proteins was detected by Western blotting (WB  $\alpha$ PAK and WB  $\alpha$ Nef, respectively).

NL4-3 Nef, together or without the dominant active p21-GTPase (Cdc42<sup>V12</sup>). In accordance with our previous data (26), when wild-type PAK2 was used, Nef-associated PAK2 activity (i.e., NAK) was only observed if Cdc42<sup>V12</sup> was cotransfected (Fig. 1, lanes 1 and 2). Thus, under the experimental conditions used here, the NL4-3 Nef allele did not substitute Cdc42<sup>V12</sup> as a PAK2 activator. Unlike wild-type PAK2, however, the constitutively active PAK2 derivatives carrying mutations in the amino-terminal regulatory region (E115K or D125R) readily associated with this Nef allele also without Cdc42<sup>V12</sup> cotransfection (Fig. 1; see also Fig. 5).

Interestingly, a very different picture was seen when the PAK2 mutant T402E was tested. In contrast to the PAK2 mutants defective in autoinhibition, PAK2-T402E failed to associate with Nef in the absence or presence of cotransfected Cdc42<sup>V12</sup> (Fig. 1, lanes 3 and 4). Thus, the molecular mechanism underlying PAK2 activation can determine whether or not PAK2 associates with Nef. Conformation associated with PAK2 activation via loss of inhibitory contacts between its N- and C-terminal domains, rather than catalytic activity per se, therefore appears to be critical for driving Nef/PAK2 complex formation.

**Nef alleles differ greatly in their capacity to stimulate PAK2 activity.** The requirement of NL4-3 Nef to precipitate NAK activity in the absence of Cdc42<sup>V12</sup> or an appropriate activating mutation in PAK2 suggested that this Nef allele was unable to stimulate PAK2 activity in the transfected cells. However, since previous studies have reported PAK activation by certain Nef alleles, such as SF2 (1), we addressed this question by examining PAK2 activity in cells transfected with PAK2 alone or together with different SIV, HIV-1, and HIV-2 Nef alleles. Figure 2 shows data on comparing NL4-3 Nef with a divergent panel of mainly primary Nef alleles. These Nef alleles have



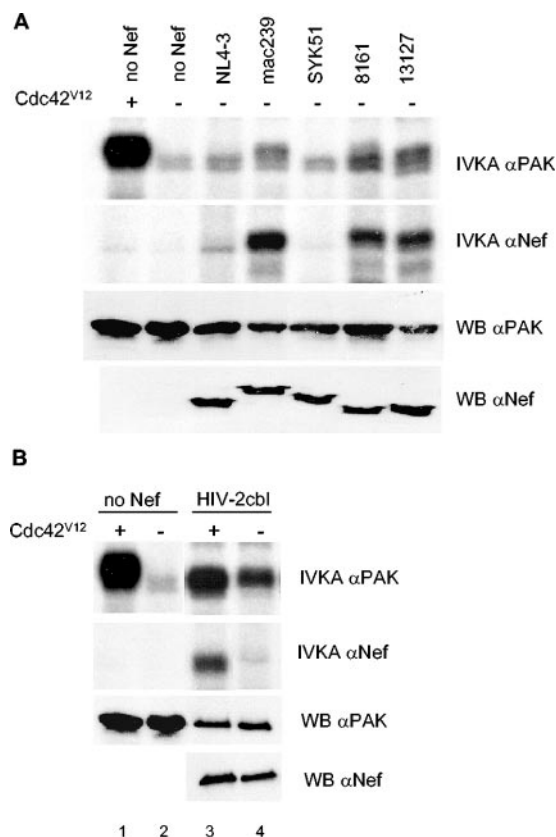


FIG. 2. (A) Capacity to stimulate PAK2 activity differs among Nef alleles. PAK2 was transfected into 293T alone (no Nef) with or without Cdc42<sup>V12</sup> or together with one of the indicated HIV-1 and SIV Nef alleles. 8161 and 13127 are both HIV-1 subtype-O Nef alleles. Total (IVKA  $\alpha$ PAK) and Nef-associated (IVKA  $\alpha$ Nef) kinase activity of PAK2 from these cells was studied as in Fig. 1. The total amount of PAK2 and Nef protein in each lysate was examined by Western blotting (WB  $\alpha$ PAK and  $\alpha$ Nef). (B) Despite a capacity to activate PAK2, HIV-2 cbl Nef depends on Cdc42<sup>V12</sup> to associate with PAK2. PAK2 alone or with HIV-2 cbl Nef was transfected into 293T cells with or without Cdc42<sup>V12</sup> and analyzed for total and Nef-associated PAK2 kinase activity as in panel A.

been recently extensively characterized for their functionality, and all were shown to be able to associate with PAK2 if cotransfected with Cdc42<sup>V12</sup> (15). As can be seen from Fig. 2A, some of these alleles were equally poor PAK2 activators, as was NL4-3 Nef, whereas others could clearly stimulate the catalytic activity of cotransfected PAK2, some being almost as efficient as Cdc42<sup>V12</sup> in this function.

This differing capacity to activate PAK2 was even more striking when NAK activity (i.e., PAK2 activity coprecipitated with Nef antibodies) in the absence of cotransfected Cdc42<sup>V12</sup> was examined. Nef alleles such as mac239, 8161, and 13127, capable of inducing kinase activity in anti-PAK2 immunocomplexes, were also associated with intense NAK activity. Thus, the capacity to stimulate PAK2 activity clearly differs greatly among Nef alleles, which is in contrast to the virtually uniform ability of divergent Nef alleles to associate and coprecipitate activated PAK2 (15).

Interestingly, however, HIV-2 cbl Nef, which was one of the most potent PAK2 activators among this panel of Nef alleles

(Fig. 2B, lane 4, of panel IVKA  $\alpha$ PAK), coprecipitated only modest amounts of NAK activity (Fig. 2B, lane 4, of panel IVKA  $\alpha$ Nef). As observed previously (15), when Cdc42<sup>V12</sup> was cotransfected HIV-2 cbl Nef efficiently precipitated NAK activity (Fig. 2B, lane 3, of panel IVKA  $\alpha$ Nef). Thus, despite its capacity to activate PAK2 HIV-2 cbl Nef remained dependent on Cdc42<sup>V12</sup> for efficient association with activated PAK2, possibly reflecting the dual role of p21-GTPases in NAK complex formation described elsewhere in this study (see discussion of the role of Cdc42 in Nef/PAK interaction below).

The different patterns of the Nef alleles tested here in stimulating cellular PAK2 activity and in associating with PAK2 clearly indicate that these are distinct and separable functions of Nef. Indeed, the failure of some Nef alleles (such as NL4-3) to stimulate PAK2 activity despite their capacity to associate with (preactivated) PAK2 demonstrates that, unlike p21-GTPases, Nef does not activate PAK2 as a direct consequence of binding.

This feature of NL4-3 Nef (its dependence for Cdc42<sup>V12</sup> or activating mutations in PAK2 in order to assemble a NAK complex) also provides a practical means to specifically study questions related to Nef/PAK2 complex assembly independently of effects of Nef on PAK2 activity, which is the experimental strategy that we have used in the studies described below.

**Nef/PAK2 complex associates with membrane rafts.** Due to myristoylation of its N terminus, a significant fraction of Nef is associated with cellular membranes (37). On the other hand, a subpopulation of cellular PAK2 becomes recruited to membranes upon activation (20, 28). Considering the dependence of Nef/PAK2 complex formation on PAK2 activation, it was therefore of interest to study the cellular compartment in which Nef/PAK2 interaction takes place. To this end, we transfected cells with NL4-3 Nef and different PAK2 variants and compared the relative amounts of PAK2 protein and PAK2 or NAK activity in the cytosolic and particulate (membrane) fractions. Notably, PAK2 activity was highly enriched in the particulate fraction in all cases, except for PAK2-T402E, whose activity was mainly cytosolic (Fig. 3, panels labeled IVKA  $\alpha$ PAK). In contrast to kinase activity, the bulk of PAK2 protein was in all cases found in the cytosol (Fig. 3, panels labeled WB  $\alpha$ PAK2). In accordance, immunoprecipitation of the same fractions with an anti-Nef-antibody detected NAK activity only in the particulate fraction (Fig. 3, panels labeled IVKA  $\alpha$ Nef). On the other hand, Nef protein was found in both fractions (Fig. 3, panels labeled WB  $\alpha$ Nef). Notably, no NAK signal was detected from the cytosolic fraction even in the case of PAK2-T402E, whose activity was mainly found in this fraction. Thus, we conclude that NAK complex is restricted to cell membranes. However, this is probably not the sole reason for the failure of PAK2-T402E to associate with Nef, because this situation was not remedied when PAK2-T402E was artificially targeted to the membranous fraction by attaching a CAAX-box element to its C terminus (data not shown).

Lipid rafts remain insoluble after treatment with 1% Triton X-100 and can be enriched to low-density fractions by flotation ultracentrifugation. Since localization of Nef in rafts has been demonstrated earlier (35), we transfected cells with the active PAK2 variant E115K with or without Nef (Fig. 4, lower or upper panels, respectively) to study whether partitioning of

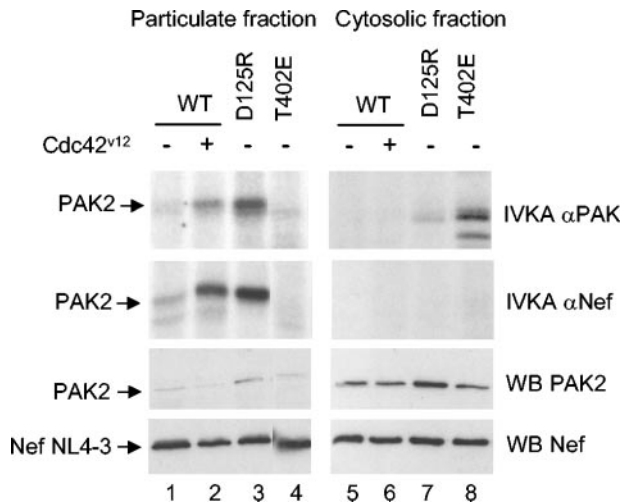


FIG. 3. Membrane localization of PAK2 and NAK activity. NL4-3 Nef and wild-type PAK2 or different activated mutants were transfected with or without Cdc42<sup>V12</sup> as indicated. Cell lysates were fractionated into particulate (left panels) and cytosolic (right panels) fractions. Part of both fractions was subjected to Western blotting analysis (two bottom panels) with antibodies against PAK2 and Nef. The rest of the fractions were used for immunoprecipitation analysis of total (IVKA αPAK) and Nef-associated (IVKA αNef) PAK2 activity as in Fig. 1.

NAK activity to the particulate fraction was due to its raft association. Successful flotation of rafts was confirmed by detection of the raft marker ganglioside GM1 by using labeled CTx (Fig. 4, panels labeled GM1). In fractionation, PAK2 protein was not enriched in rafts and instead was detected in all fractions, including the bottom ones (fractions 5 to 8 in Fig. 4) in accordance with its mainly cytosolic localization shown in Fig. 3. In contrast, PAK2 activity was enriched in the GM1-containing fractions and less abundant in the bottom fractions. However, raft association of PAK2 activity was much less striking than its highly selective association with the membrane fraction (Fig. 3), indicating that at least two-thirds of PAK2 activity is associated with membranes other than rafts. A similar but more pronounced raft association of PAK activity was recently reported by Krautkramer et al. (17). Their data also suggested a marked role for Nef in recruiting PAK to rafts, which was not evident in our experiments.

Interestingly, however, NAK activity was exclusively found in fractions 1 and 2 (Fig. 4B, panel labeled IVKA αNef), suggesting that NAK activity is more selectively associated with rafts than is total PAK2 activity. In agreement with previously published data (35, 39), a subpopulation (estimated 20%) of Nef protein localized to rafts but was also found in other fractions (Fig. 4, panel labeled WB αNef). Thus, the selective localization of the NAK complex to the rafts cannot be explained simply by the lack of Nef and PAK2 in other subcellular compartments.

**Role of Cdc42 in Nef/PAK2 interaction.** The Cdc42/Rac-binding domain (CRIB) connects PAKs to these p21-GTPases, and mutations introduced to CRIB abolish p21-mediated stimulation of PAK activity (38). We have previously noticed that leucine substitution of two critical histidine residues (H82 and H85) in the CRIB domain of PAK2 (mutant dubbed PAK2-

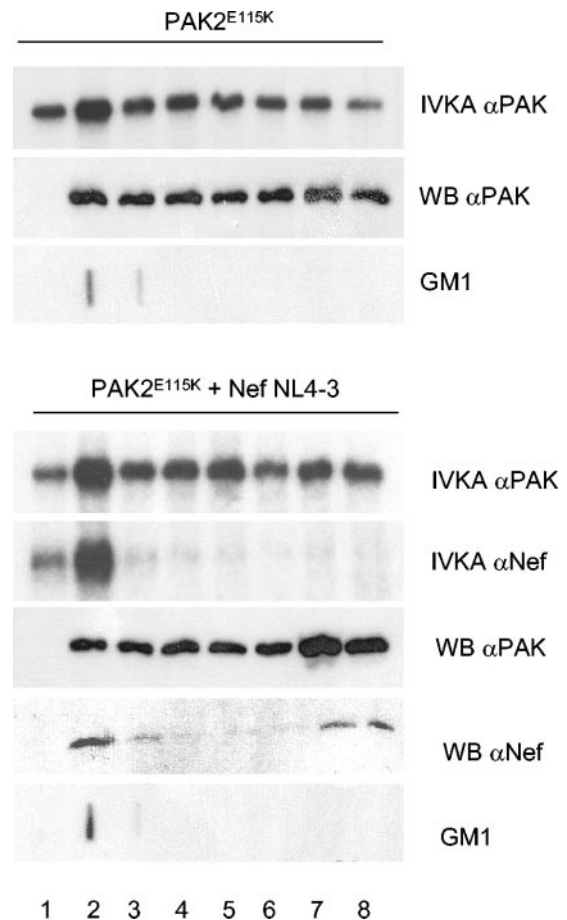


FIG. 4. Nef associates with PAK2 in a raft-containing membrane population. Lysates of cells transfected with PAK2-E115K with or without Nef NL4-3 were subjected to membrane flotation on Opti-prep gradient centrifugation. Eight fractions (starting from top; lanes 1 to 8) were collected and analyzed for total (IVKA αPAK2) and Nef-associated (IVKA αNef) PAK2 activity as in Fig. 2. PAK2 and Nef protein expression in these fractions was examined by Western blotting (WB αPAK and WB αNef), whereas the raft marker GM1 present in these fractions was detected by slot blotting and visualization with labeled CTx subunit B (GM1).

HH) prevented Nef/PAK2 complex formation (26). Therefore, a question arises whether the CRIB motif dependence of NAK complex is due to lack of p21-mediated PAK2 activation or a direct consequence of the loss of Cdc42<sup>V12</sup>/PAK2 interaction.

To address this issue, we first constructed another p21-binding deficient PAK2 mutant containing amino acid substitutions in three other critical CRIB motif residues (I74N;S75P;P77A [38], mutant dubbed PAK2-ISP). PAK2-ISP failed to associate with Nef (Fig. 5A, bottom panel, lanes 3 to 6), confirming the observation made with PAK2-HH and therefore supporting the idea that these mutations probably affected Nef association by PAK2 specifically by preventing p21 binding. Because the constitutively active PAK2-E115K and PAK2-D125R could associate with NL4-3 Nef without Cdc42<sup>V12</sup> cotransfection (Fig. 1 and 5), we created PAK2 double mutants containing both a conformationally activating mutation (E115K or D125R) and a CRIB-inactivating mutation (HH or ISP). However, even when combined with D125R or E115K mutation, HH- and

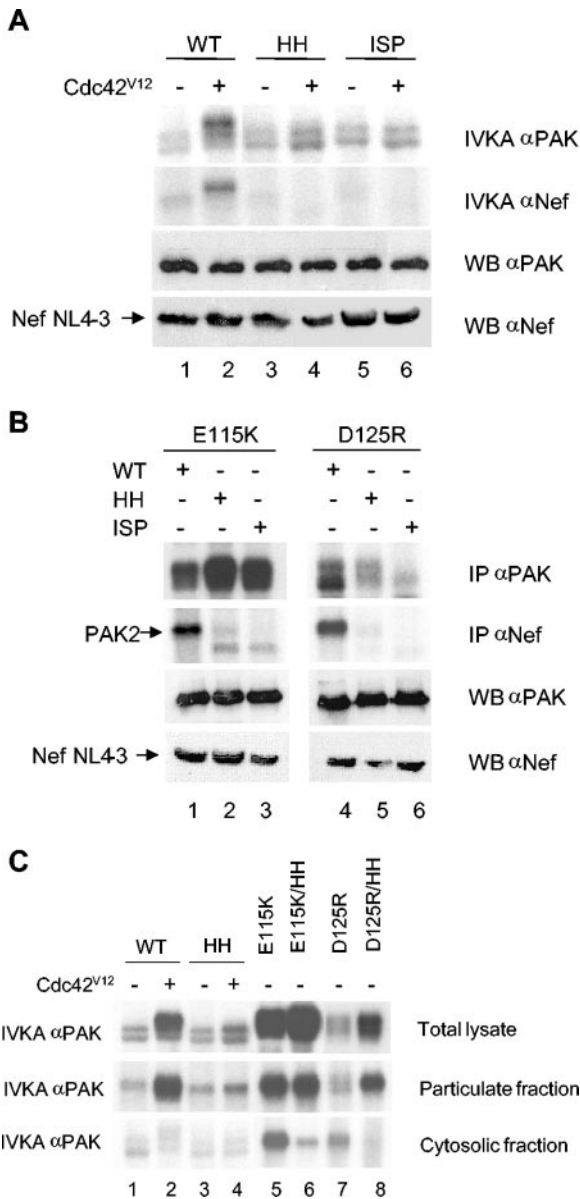


FIG. 5. Cdc42-binding in the association of PAK2 with Nef and localization to the particulate fraction. (A) CRIB domain of PAK2 is essential for Nef association. Nef and wild-type (WT) PAK2 or different Cdc42-binding deficient mutants, PAK2-HH or PAK2-ISP, were transfected, together with Cdc42<sup>V12</sup>, where indicated. Total PAK2 activity (top panel) and Nef-associated PAK2 activity (bottom panel) were studied by anti-Myc (for PAK2) or anti-Nef immunoprecipitations and IVKA. Expression of PAK2 and Nef proteins was studied by Western blotting (WB αPAK and WB αNef). (B) Release of autoinhibition does not substitute for Cdc42-binding deficiency for association with Nef. Total PAK2 activity (top panel) and Nef-associated PAK2 activity were studied as in panel A from cells that were transfected with Nef and PAK2-E115K or PAK2-D125R either with a functional (WT) or mutated (HH or ISP) CRIB motif. (C) Membrane localization of PAK2 is Cdc42-binding independent. Wild-type (WT) PAK2, different activated mutants (E115K and D125R), or Cdc42-binding-deficient mutants (HH, E115K/HH, and D125R/HH) were transfected. Total cell lysates, as well as the particulate and cytosolic fractions, were collected from these cells, and the total PAK2 activity was studied from both fractions as in panel A.

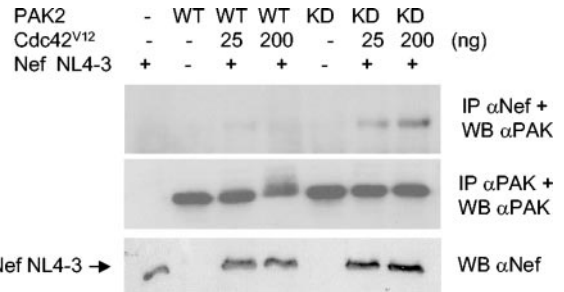


FIG. 6. Kinase activity of PAK2 destabilizes the NAK complex. Nef and wild-type (WT) PAK2 or the kinase-dead (KD) PAK2-K278R variant were cotransfected, together with increasing amounts of Cdc42<sup>V12</sup> as indicated. Lysates were immunoprecipitated with anti-Nef or anti-PAK2 antibodies, followed by anti-PAK2 Western blotting to visualize Nef-associated (IP αNef) and total (IP αPAK) PAK2. Expression of total Nef protein in each lysate was examined by Western blotting (WB αNef).

ISP-mutated PAK2 variants still failed to associate with Nef (Fig. 5B, lanes 2 and 3 and lanes 5 and 6). Since the formation of the Nef/PAK2 complex was found to be restricted to cellular membranes, the failure of the E115K/HH and D125R/HH combination mutants to associate with Nef could simply reflect their inability to localize to cell membranes. When the subcellular localization of these double mutants was examined, however, their activity was found to be equally or even more selectively membrane-associated compared to E115K and D125R with intact CRIB motifs (Fig. 5C, lanes 5 to 8).

These data show that a functional CRIB domain is required for NAK complex formation for reasons that cannot be assigned only to its enabling p21-mediated activation of PAK2. A plausible explanation for this observation would be that a p21-GTPase is physically involved in stabilization of the Nef/PAK2 complex. Since PAK2-E115K and PAK2-D125R with an intact CRIB motif could associate with NL4-3 Nef without Cdc42<sup>V12</sup> cotransfection, enough endogenous active p21-GTPases appear to be present in the lipid rafts of these cells to help in stabilizing the NAK complex, although this low p21-GTPase activity is insufficient to drive activation and membrane relocalization of native PAK2.

**Kinase activity of PAK2 promotes disassembly of the Nef/PAK2 complex.** Nef efficiently associates with the catalytically active subpopulation of PAK2, but this represents only a small fraction of total cellular PAK2 protein (1, 26). As a consequence, sensitive immunocomplex phosphorylation assays (Fig. 1 to 5) have until now been the only practical means to study the NAK complex. The notion that a p21-GTPase may be directly involved in Nef/PAK2 complex formation (Fig. 6) led us to hypothesize that this interaction might take place during the process of PAK2 activation and fall apart after the kinase activity of PAK2 has been induced.

To test this idea, we examined whether a kinase-deficient mutant of PAK2 (K278R) could associate more stably with Nef than wild-type PAK2. Indeed, by cotransfecting increasing amounts of Cdc42<sup>V12</sup>, together with PAK2-K278R, a corresponding increase in PAK2 coprecipitation with Nef could be observed by Western blotting (Fig. 6). This result supports the idea that catalytic activity of PAK2 is involved in destabilizing the NAK complex and shows that using a kinase-defective



PAK2 results in accumulation of a sufficient amount of Nef/PAK2 complex in cells and that this interaction can be visualized by a traditional coprecipitation/immunoblotting approach. In contrast, the amount of wild-type PAK2 coprecipitating under these conditions was too small to be consistently detected, even when the highest amount (200 ng) of Cdc42<sup>V12</sup> expression vector was cotransfected. Attempts to increase the association between wild-type PAK2 and Nef by transfecting even larger amount of Cdc42<sup>V12</sup> have not been successful and have instead resulted in substantially decreased PAK2 protein levels (data not shown), possibly through increased PAK2 degradation and/or cytotostasis (10).

## DISCUSSION

We have previously reported that Nef selectively associates with catalytically active PAK2, but the role of Nef-induced PAK activation in assembly of the NAK complex has remained unclear. NAK activity in anti-Nef immunocomplexes has been commonly interpreted as evidence of a capacity of Nef to stimulate PAK activity, but few studies have directly addressed this issue. Recently, Arora et al. reported that an increase in PAK2 kinase activity could be demonstrated in SF2 Nef-transfected cells compared to control-transfected cells (1). However, results of similar experiments by us with the NL4-3 Nef allele (26) (Fig. 1) have been negative and less than convincing when SF2 Nef was used (unpublished data). By testing a panel of diverse Nef alleles we show here that Nef should indeed be considered a PAK2 activator, but this property is weak or lacking in many Nef alleles available for study. Most of the Nef alleles that showed potent PAK2 activation were molecular clones isolated without passage of the virus in cell culture. Although this correlation is not sufficient for drawing any conclusions, it is possible that potent PAK2 activation by Nef might for some reason be counterselected during *in vitro* growth. In any case, given that some Nef alleles clearly have a capacity to stimulate PAK2 activity, the differing results obtained with “weaker” alleles may simply have reflected differences in assay sensitivity.

Unlike the varying capacity of Nef alleles to promote PAK2 kinase activity, the ability to associate with active PAK2 is a highly conserved Nef function (15, 26, 31). Therefore, a failure to stimulate PAK2 activity rather than a failure to associate with the NAK complex probably explains the lack of serine/threonine kinase activity reported to be associated with some Nef alleles. The segregation of these properties among different Nef alleles also implicates that these two Nef functions are mechanistically distinct. Thus, Nef is not mimicking an active p21-GTPase, which acts by pushing PAK2 into an active conformation via binding to its CRIB motif. Instead, Nef appears to act upstream of the p21-GTPase to promote their capacity to stimulate PAK2 activation. This scenario agrees with the model proposed based on studies from the Peterlin laboratory, where Nef acts as an adaptor to recruit proteins, including phosphatidylinositol 3-kinase and Vav into a “signalosome” in which PAK activation could occur (9, 19, 24). On the other hand, an alternative model was recently proposed by a study from the Skowronski laboratory, which reported that Nef stimulates cellular guanine nucleotide exchange factor activity and consequently p21-GTPase-regulated signaling cascades by as-

sociating with the DOCK2/ELMO1 complex (12). Of course, these two models need not to be mutually exclusive, and Nef might possess redundant strategies for stimulating cellular p21-GTPases.

When the terms “upstream” and “downstream” are used to describe effects of Nef on cellular signaling, it is important to note that they refer to the order of events but do not imply that these processes would have to be spatially separated. Indeed, the dual role of Cdc42<sup>V12</sup> revealed by the present study suggests that, although PAK2 activation and association are mechanistically distinct functions of Nef, they take place in the same multiprotein complex. Specifically, we found that, apart from enabling conformational activation of PAK2, its CRIB motif served an additional important role in the Nef/PAK2 complex. This suggested that an active p21-GTPase is also physically involved in stabilizing the NAK complex. Moreover, the idea that PAK2 activation and association by Nef involve a common protein assembly is also supported by the fact that Nef-induced increase in cellular p21-GTPase activity results in selective activation of PAK2 but not PAK1 (1) [our unpublished data], although Cdc42 and Rac1 have no intrinsic preferences as activators of these two PAK family kinases.

Our fractionation studies revealed that the NAK complexes are restricted to membrane domains known as lipid rafts. Such raft specificity is striking, since active PAK2 as well as Nef could also be found in most other membrane fractions. This observation might be explained by the critical role of lipid rafts in regulation of the cellular activity of the p21-GTPase Rac1 (6). Although active PAK2 may leave these membrane domains, the role of an active (GTP-bound) p21-GTPase in stabilizing the Nef/PAK2 interaction could account for restriction of this complex to rafts.

Another factor that was found to contribute to limiting the NAK activity close to its origin, i.e., the membrane-associated p21-GTPase-containing activation complex, was the apparent destabilizing effect of PAK2 kinase activity on the NAK complex. This suggested a model where a p21-GTPase is required for assembly of the NAK complex, but CRIB-mediated catalytic activation of PAK2 subsequently promotes rapid dissociation of this complex. This dynamic and transient nature of the NAK complex could also explain its low abundance even when Nef and PAK2 are overexpressed in cells. This scenario was suggested by the observation that cotransfection of Nef and Cdc42<sup>V12</sup> together with a kinase-deficient PAK2 mutant (K278R) led to a significantly more stable Nef/PAK2 complex. Indeed, Nef/PAK2-K278R interaction could be readily demonstrated by a standard coimmunoprecipitation and Western blot procedure, which has been difficult in the case of a regular NAK complex. This approach should provide new experimental opportunities for elucidation of the functional role of the Nef/PAK2, for example, by facilitating proteomics studies aimed at complete characterization of the proteins required for the assembly and involved in effector functions of the NAK complex.

Despite its constitutive catalytic activity PAK2-T402E differed starkly from PAK2 activated by mutations that mimic the effect of p21 binding (E115K and D125R) in that it failed to associate with Nef as well as with cell membranes. We speculate that such direct activation of the PAK2 catalytic apparatus by the T402E mutation may result in a conformation/autophos-

phorylation pattern that differs from PAK2 activated via a physiological p21-GTPase-mediated mechanism. As a consequence of its aberrant conformation PAK2-T402E might be unable to support an interaction(s) that normally couples activated PAK2 to its membrane-associated partners. Whatever the reason, the strikingly different biological behavior of these two different types of dominantly active PAK2 mutants has important implications for future research on Nef/PAK2 interaction, as well as on PAK family kinases in general. Although it is obvious that only PAK2-E115K and PAK2-D125R but not PAK2-T402E can be used as a “dominant active NAK,” our data also suggest that the choice of the activating mutation is also likely to profoundly influence studies on other aspects of PAK in cell biology.

Protection from cell death via NAK-mediated phosphorylation and inactivation of the proapoptotic Bad protein has been described as an important function of Nef, which could in part explain its pathogenic effect in HIV infection (36). NAK has also been implicated as an effector of Nef in increasing HIV gene expression and virion production in the infected cells (8, 32) and thus represents a potentially useful therapeutic target. Detailed understanding of the composition and function of the NAK multiprotein complex could allow the design of compounds that would target a cellular (and hence nonmutating) factor and thereby specifically block Nef-mediated PAK2 activation without interfering with the normal functions of this important kinase.

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# Human Immunodeficiency Virus Type 1 Nef Recruits the Guanine Exchange Factor Vav1 via an Unexpected Interface into Plasma Membrane Microdomains for Association with p21-Activated Kinase 2 Activity<sup>∇</sup>

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**Alterations of T-cell receptor signaling by human immunodeficiency virus type 1 (HIV-1) Nef involve its association with a highly active subpopulation of p21-activated kinase 2 (PAK2) within a dynamic signalosome assembled in detergent-insoluble membrane microdomains. Nef-PAK2 complexes contain the GTPases Rac and Cdc42 as well as a factor providing guanine nucleotide exchange factor (GEF) activity for Rac/Cdc42. However, the identity of this GEF has remained controversial. Previous studies suggested the association of Nef with at least three independent GEFs, Vav, DOCK2/ELMO1, and βPix. Here we used a broad panel of approaches to address which of these GEFs is involved in the functional interaction of Nef with PAK2 activity. Biochemical fractionation and confocal microscopy revealed that Nef recruits Vav1, but not DOCK2/ELMO1 or βPix, to membrane microdomains. Transient RNAi knockdown, analysis of cell lines defective for expression of Vav1 or DOCK2 as well as use of a βPix binding-deficient PAK2 variant confirmed a role for Vav1 but not DOCK2 or βPix in Nef's association with PAK2 activity. Nef-mediated microdomain recruitment of Vav1 occurred independently of the Src homology 3 domain binding PxxP motif, which is known to connect Nef to many cellular signaling processes. Instead, a recently described protein interaction surface surrounding Nef residue F195 was identified as critical for Nef-mediated raft recruitment of Vav1. These results identify Vav1 as a relevant component of the Nef-PAK2 signalosome and provide a molecular basis for the role of F195 in formation of a catalytically active Nef-PAK2 complex.**

The Nef protein of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) is a multifunctional factor that is critical for high virus titers in vivo. Consequently, disease progression in individuals infected with *nef*-deficient viruses is very slow or absent (16, 30, 32). These effects are thought to mirror several independent activities of Nef that prevent immune recognition of virally infected cells and boost HIV replication (18, 36, 57, 72). In order to achieve such an optimized spread in the infected host, Nef manipulates a variety of transport and signal transduction processes in cells infected by HIV type 1 (HIV-1). Modulation of cellular transport pathways by Nef affects the surface presentation of a large number of cell surface receptors, such as CD4, major histocompatibility complex class I and II molecules, and chemokine receptors, to prevent superinfection and to facilitate immune evasion of productively infected cells (8, 15, 22, 45, 65, 70). Equally widespread are Nef effects on host cell signaling, including various alterations of the T-cell receptor (TCR) cascade in T lymphocytes (6, 7, 40, 43, 44, 59, 61, 64, 66, 68). Since resting T lymphocytes are largely resistant to productive HIV infection, activation of target T lymphocytes is a prerequisite for efficient spread of HIV-1 in vivo (reviewed in reference 69).

At the same time, high activation levels induced by potent extracellular stimulation drive cells into activation-induced cell death. According to an emerging view, Nef acts as an intracellular inducer of TCR-triggered distal signaling events in the absence of exogenous stimulation, while signaling via TCR from outside of the cell is tuned down by Nef (21, 27, 62, 71; see reference 17 for a review). By balancing these mechanisms, Nef may thus ensure both target cell permissiveness and survival for optimal virus production.

Nef alters host cell processes by interacting with components of cellular machines. Previous studies have identified a number of protein interaction surfaces in Nef that are vital for specific activities of the viral protein (3, 4, 23). Since an increasing number of host cell factors that interact with each of these Nef interfaces have been reported, the functional relevance of individual ligands have remained largely elusive. Regarding its signal transduction properties, Nef is known to associate with a number of cellular kinases, including members of the protein kinase C, Src tyrosine, and p21-activated kinase (PAK) kinase families (reviewed in reference 56). In particular, the association of Nef with PAK has been studied in more detail, demonstrating that PAK2 is the preferred PAK isoform that associates with Nef (5, 19, 54). The interaction of Nef with PAK2 activity is conserved for many Nef proteins derived from HIV-1, HIV-2, and SIV strains (50, 51, 60) and is readily detectable in HIV-1-infected T lymphocytes as well as HIV-1 provirus transgenic mice (59, 75). Many Nef alleles also aug-

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ment overall cellular PAK2 activity, but PAK2 activation is less well conserved and dispensable for the association of Nef with PAK2 activity (51). Some studies using Nef mutants that fail to associate with PAK2 activity but are also defective in other Nef functions concluded that the Nef-PAK2 association may contribute to the elevated pathogenic potential of Nef-positive viruses *in vivo* (31, 61, 75). These reports are at odds with a similar study in which viruses encoding a Nef mutant with disruption of an interaction motif critical for Nef's association with PAK2 activity caused acute disease (35). If relevant for the pathogenic potential of lentiviruses, the underlying mechanism of the Nef-PAK2 association is not well understood but might involve established consequences such as upregulation of HIV transcription, remodeling of the actin cytoskeleton, prevention of apoptosis, and enhancement of virion infectivity (11, 19, 20, 27, 40, 76, 77). Thus, as for most protein interactions of Nef, the exact role and relevance of the Nef-PAK2 complex for virus propagation in the infected host remain to be defined.

The Nef-PAK2 association occurs in the context of a larger signalosome of approximately 1 MDa in size (19). Assembly of this complex occurs at cellular membranes, where it selectively segregates in detergent-resistant microdomains of the plasma membrane, and disruption of these microdomains potentially blocks Nef's association with PAK2 activity (34, 51, 53). The association of Nef with the kinase is therefore mediated selectively by a small subpopulation that associates with these microdomains (24, 34, 51). While it has been demonstrated that Nef recruits PAK2 to membrane microdomains similarly to the physiological PAK2 activators Rac1 and Cdc42 (34, 51, 53), further analysis of the composition of the Nef-PAK2 complex and its regulation has been hampered by its low stability. Although Nef-associated PAK2 activity can readily be demonstrated experimentally, Western blot detection of PAK2 protein in Nef immunoprecipitates has been challenging (2, 40, 48, 51, 59, 61). This may reflect the specific association of Nef with a highly active PAK2 subpopulation whose activity triggers rapid disassembly of the complex (51, 55).

More detailed information is available on the determinants in Nef that govern assembly of the Nef-PAK2 signalosome. Consistent with its membrane microdomain localization, Nef's N-terminal myristoylation, a prerequisite for its association with membranes, is essential for the functional interaction with PAK2 (60). Earlier studies also identified a di-arginine motif as a critical determinant. Mutation of this motif, however, has pleiotropic effects, including decreased protein stability, and it has thus been questioned whether this interface is actively involved in Nef-PAK2 complex formation (23, 49, 60). Recently, a novel protein interaction surface in Nef surrounding the key residue F195 was identified as essential for the association of Nef with active PAK2 (1, 2, 49). One study suggested that this interface facilitates the recruitment of PAK2 into the complex; the molecular basis for this role of F195, however, has not been addressed in more detail (2). Finally, Nef contains a highly conserved, proline-rich Src homology 3 domain binding (PxxPxR) motif that represents another key determinant for assembly of an enzymatically active Nef-PAK2 complex (31, 42, 76). Since functional Nef-PAK2 association depends on the activity of the upstream small GTPases Rac1 and Cdc42 (40, 48), it has been assumed that an SH3-mediated

interaction by Nef directly or indirectly recruits a guanine nucleotide exchange factor (GEF) to ensure PAK2 activity within the complex. The identity of this GEF, however, has remained a matter of debate. With Vav1,  $\beta$ Pix/Cool, and DOCK2/ELMO1, at least three GEFs containing an SH3 domain and providing GEF activity toward Cdc42 and/or Rac were reported to associate with Nef (9, 20, 28, 67). Vav1 was previously suggested as a critical component for the functional association of Nef and PAK2; this was based, however, exclusively on overexpression of a dominant-negative variant (20). Conflicting data exist on the role of  $\beta$ Pix in the Nef-PAK2 signalosome (9, 55, 75), and DOCK2/ELMO1 has not been analyzed with respect to Nef's association with active PAK2 (28). To address which of these GEFs play functional roles in the Nef-PAK2 complex, their recruitment into membrane microdomains by Nef and the effects of reduced GEF expression levels upon specific RNA interference (RNAi) or genetic knockout on the efficiency of the Nef-PAK2 association were used in this study as independent criteria. Our results demonstrate the involvement of Vav1 in the association of Nef with PAK2 activity and unexpectedly reveal that this GEF is recruited into the complex via the F195 surface rather than the PxxPxR motif.

#### MATERIALS AND METHODS

**Cells, reagents, and plasmids.** Jurkat Tag (JTag), JVav, and BE $\alpha$ 16-3 cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin (all from Invitrogen). JVav and BE $\alpha$ 16-3 cells are Jurkat derivatives and T-cell hybridoma cells that lack Vav1 or DOCK2 expression (12, 58), respectively, and were kindly provided by Daniel Billadeau and Yoshinori Fukui. Expression constructs for Vav1.myc, green fluorescent protein (GFP), various Nef.GFP proteins, PAK2, and  $\beta$ Pix binding-deficient Pak2 proteins were described elsewhere (13, 20, 25, 27, 34). Nef from HIV-1 SF2 was used throughout. Constructs for expression of Nef.GFP carrying the F195A or F195I mutation were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene), and the Nef-coding sequences were verified by sequencing. Expression plasmids for  $\beta$ Pix.myc, Flag-DOCK2, and ELMO1.His were kindly provided by Ivan Dikic, Shinya Tanaka, and Yoshinori Fukui, respectively (47, 58, 63).

The following antibodies were used: polyclonal rabbit anti-ELMO1, polyclonal rabbit anti-Vav2, and monoclonal mouse anti-myc (clone 9E10) (all obtained from Santa Cruz Biotechnology); monoclonal mouse anti-flag (clone M2), monoclonal mouse anti-GFP (clone GFP20), and polyclonal mouse anti-cholera toxin (anti-CTx) (all from Sigma-Aldrich); polyclonal rabbit anti-PAK1/2/3 and polyclonal rabbit anti-Vav (both from Cell Signaling Technology); polyclonal rabbit anti- $\beta$ PIX and monoclonal mouse anti-His<sub>6</sub> (clone BMG-His-1) (Roche); polyclonal rabbit anti-linker of activated T cells (LAT) (Upstate Biotechnology); and monoclonal mouse anti-transferrin receptor (anti-Tfr) (clone H68.4) (Zymed Laboratories, Inc.). Secondary fluorescent antibodies and Alexa Fluor 555-conjugated CTx subunit B were obtained from Molecular Probes, and protease inhibitor cocktail was purchased from Sigma. Polyclonal rabbit serum against GFP was kindly provided by Hans-Georg Krüsslich, and polyclonal sheep serum against Nef was a kind gift from Mark Harris (14).

**Western blotting.** For Western blot analysis, samples were boiled in sodium dodecyl sulfate (SDS) sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. Protein detection was performed following incubation with appropriate first and secondary antibodies using the Super Signal Pico detection kit (Pierce) according to the manufacturer's instructions.

**IVKA.** Nef-associated PAK2 activity was analyzed in an *in vitro* kinase reaction following immunoprecipitation of Nef.GFP essentially as described previously (24, 34). Cells were transfected with the respective plasmids and RNAi oligonucleotides via electroporation and incubated for 24 h (BE $\alpha$ 16-3 and JVav cells), 48 h (PAK1 RNAi), or 72 h (all other RNAi constructs). For this, 15 to 50  $\mu$ g DNA and 500 pmol of RNAi specific for the targeted sequences, or unspecific as a control, were added to  $1 \times 10^7$  cells in 4-mm cuvettes, and electroporation took place at 250 V (JTag and JVav) or 230 V (BE $\alpha$ 16-3), 950  $\mu$ F, in a GenePulser

Xcell (Bio-Rad). Sequences targeted by RNAi were 5' UCU GUA UAC ACA CGG UCU GTT 3' for PAK1, 5' AGA AGG AAC UGA UCA UUA ATT 3' for PAK2, 5' CGU CGA GGU CAA GCA CAU UTT 3' for Vav1, 5' AGU CCG GUC CAU AGU CAA CTT 3' for Vav2, 5' ACC ACU GUC UGC AAU AAU ATT 3' for ELMO1, 5' GGA ACG ACA UCU ACA UUA CTT 3' for DOCK2, 5' GGA UGA AGU UCA AGA AUU ATT 3' for  $\beta$ Pix, and 5' AGG UAG UGU AAU CGC CUU GTT 3' as an unspecific control (all from MWG-Biotech). Cells were lysed in KEB (137 mM NaCl, 50 mM Tris HCl [pH 8], 2 mM EDTA, 0.5% Nonidet P-40,  $\text{Na}_3\text{VO}_4$ , and protease inhibitors) and subjected to immunoprecipitation using rabbit anti-GFP serum as described (27). After extensive washing in KEB, beads were resuspended in 50  $\mu$ l KAB (50 mM HEPES [pH 8], 150 mM NaCl, 5 mM EDTA, 10 mM  $\text{MgCl}_2$ , 0.02% Triton X-100). Treatment with 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP (5 min, room temperature [RT]) allowed the detection of PAK2 autophosphorylation. Following extensive washing in KEB, *in vitro* kinase assay (IVKA) reaction products were separated by SDS-polyacrylamide gel electrophoresis and blotted on a nitrocellulose membrane. Radioactive signals were visualized and quantified with a PhosphorImager (Bio-Rad). Immunoprecipitated proteins were detected by Western analysis using anti-GFP antibodies and quantified using the QuantityOne software (Bio-Rad). Radioactive signals were normalized against the amount of isolated Nef in the Western blot, and signals of wild-type (wt) Nef.GFP were arbitrarily set to 100%. Statistical significance was determined by Student's *t* test.

**Isolation of DRMs.** Detergent-resistant membranes (DRMs) were isolated by flotation experiments as described previously (34). In brief, JTag cells were transfected via electroporation as described for the IVKA and incubated for 48 h. A total of  $4 \times 10^7$  cells were transfected for each sample. Cells were lysed in 400  $\mu$ l TXNE buffer (1% Triton X-100, 50 mM Tris HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA) for 20 min on ice, homogenized with a Dounce homogenizer, and loaded on an Optiprep (Life Technologies) gradient (lysates were adjusted to 40% Optiprep and overlaid with 2.5 ml 28% Optiprep and 600  $\mu$ l TXNE), followed by ultracentrifugation (35 000 rpm, 3 h, 4°C). Eight fractions of 500  $\mu$ l each were collected from the top. For further analysis, fraction 2, which represented the DRM fraction, and fraction 8, which was indicative of the soluble fraction, were used in a Western blot. The quality of the flotation was addressed by using Tfr (excluded from DRMs) and LAT (incorporated in DRMs).

**CTX clustering.** The generation of CTx-positive clusters (CTx clusters) was performed as described before (34). A total of  $1 \times 10^7$  JTag cells were transfected via electroporation and incubated with 25  $\mu$ g of Alexa 555-conjugated CTx/ml in 0.1% bovine serum albumin-phosphate-buffered saline for 30 min at 4°C, followed by addition of anti-CTX antibody (1:200; 30 min at 4°C and 10 min at 37°C). Cells were bound to poly-L-lysine-coated coverslips (5 min, RT), fixed with 3% paraformaldehyde (10 min, RT), and extracted with 0.1% Triton X-100 (1 min, RT). Cells were stained using the respective anti-tag antibodies (1:50 anti-His and 1:500 anti-flag and anti-myc for 1 h), followed by Alexa 660-conjugated secondary antibody (1:2,000 for 30 min at RT), and were mounted in Histomount (Linaris). Samples were analyzed in a confocal laser scanning microscope (LSM 510; Zeiss) using a 100 $\times$  oil immersion objective lens. Images were processed with Adobe Photoshop.

## RESULTS

**Nef-mediated recruitment of Vav1 to membrane microdomains.** The aim of this study was to evaluate the relative involvement of three cellular GEFs, Vav,  $\beta$ Pix, and DOCK2/ELMO1, which have been reported to interact functionally or physically with the Nef/PAK2 complex. Since the Nef-PAK2 association is confined exclusively to the detergent-insoluble fraction in microdomain flotation experiments and microdomain association of Nef correlates with its ability to associate with PAK2 (24, 34, 51), we used the presence in such microdomains as the first criterion to define a GEF relevant for the Nef-PAK2 complex. In order to allow parallel biochemical and microscopic analyses, Nef.GFP fusion protein from the HIV-1 strain SF2 was used. This fusion protein has previously been shown to be functionally equivalent to native Nef (24, 27, 34). Using previously validated experimental procedures (24, 34), microdomain association was first assessed by flotation analysis of DRMs after Triton X-100-based lysis at 4°C of transiently

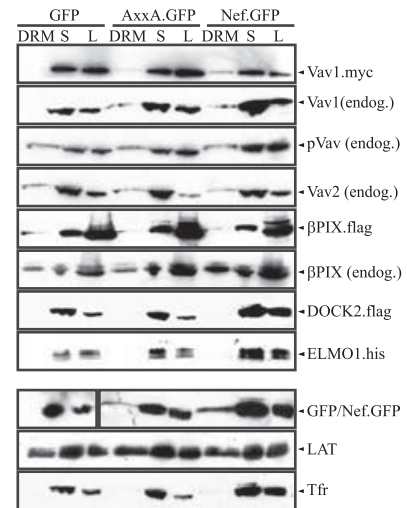


FIG. 1. Analysis of Nef-mediated DRM recruitment upon transient expression of cellular GEFs. Membrane microdomain flotation analysis from JTag T lymphocytes transiently expressing different Nef.GFP fusion proteins or GFP is shown. Cell lysates (1% Triton X-100) were separated by Optiprep gradient ultracentrifugation, and eight fractions were collected from the top (fraction 1) to the bottom (fraction 8) of the gradient. The DRM (fraction 2) and the soluble fraction (S) (fraction 8) were analyzed together with the unfractionated cell lysate (L) by Western blotting for the distribution of GFP/Nef.GFP and the GEF. Tfr and LAT were analyzed as markers for S and DRM fractions, respectively. For DRM and S, 4% of each fraction was loaded; L corresponds to 1% of the total lysate. When indicated, expression constructs of epitope tagged GEFs were cotransfected with GFP/Nef.GFP. The results presented are representative of at least four independent experiments.

transfected JTag T lymphocytes. Effects of Nef on GEF segregation were assessed by comparing cells expressing Nef.GFP, a GFP control, or a Nef.GFP mutant protein that fails to associate with active PAK2 due to disruption of its SH3 domain-interacting motif (AxxA.GFP). In addition to expression plasmids for GFP/Nef.GFP, either these cells were cotransfected with expression constructs for epitope-tagged GEFs or endogenous GEFs were analyzed. Following microdomain flotation, equal volumes of DRM and soluble fractions as well as total cell lysate were analyzed by Western blotting (Fig. 1). Exclusion of Tfr from the DRM fraction and the nearly equal distribution of LAT between the DRM and soluble fractions served as quality controls for the DRM isolation procedure.

As expected, soluble GFP was excluded from DRMs while Nef.GFP was detected in small but significant amounts in DRMs. In comparison, the AxxA.GFP mutant was expressed at somewhat reduced levels, as previously noted (13). While these lower levels of expression resulted in relatively small absolute amounts of DRM association, the distributions between the DRM and soluble fractions were approximately similar for Nef.GFP and AxxA.GFP. Coexpressed Vav1 was undetectable in DRMs in GFP-expressing control cells. In contrast, detectable amounts of the GEF were present in DRMs upon coexpression of Nef.GFP, suggesting recruitment of Vav1 by Nef. Consistent results were obtained when endogenous Vav1 was analyzed. Surprisingly, Vav1 was also enriched in DRMs in the presence of AxxA.GFP. Indeed, when taking the reduced relative expression levels and specific DRM local-



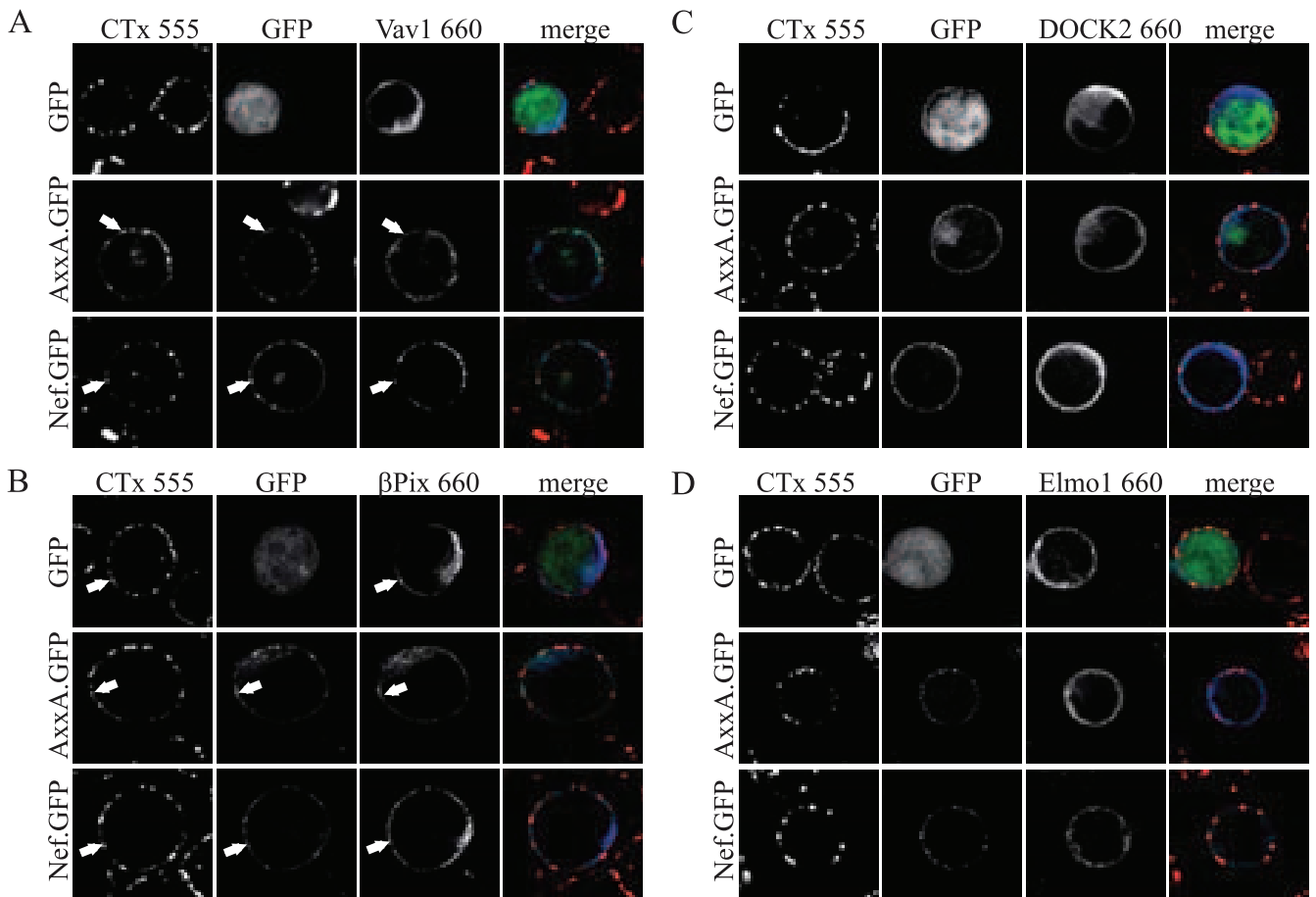


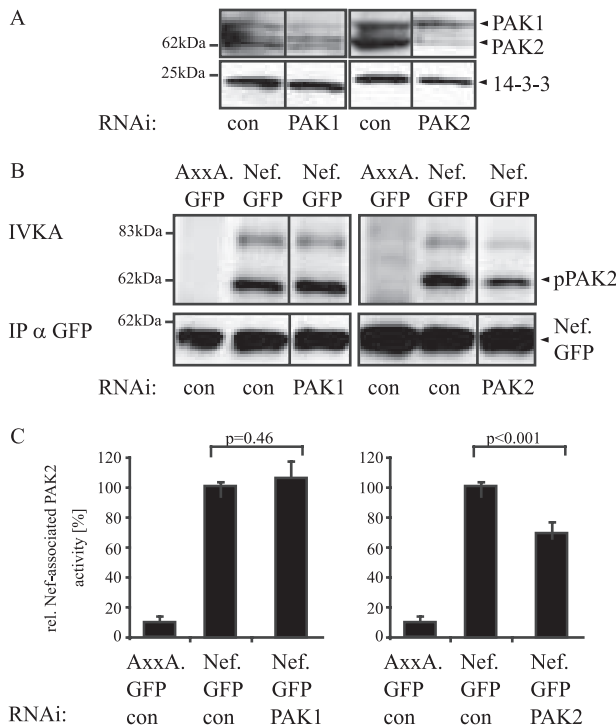
FIG. 2. Analysis of Nef-mediated recruitment of cellular GEFs into plasma membrane microdomains. Aliquots of the transfected JTag T lymphocytes analyzed in Fig. 1 were subjected to raft clustering by incubation with CTx conjugated with Alexa 555 fluorescent dye and subsequently cross-linked with anti-CTx antibody. The GEFs were stained with anti-tag antibodies and detected with Alexa 660 coupled to the appropriate secondary antibodies. Cells were analyzed by confocal microscopy, and single representative sections are presented. The merge panels depict the overlay of all three fluorescence channels, with GFP/Nef.GFP in green, CTx in red, and the respective GEF in blue. Arrows indicate colocalization of Nef with a GEF in plasma membrane microdomains. The results presented are representative of at least three independent experiments.

ization of this Nef mutant into account, DRM recruitment by AxxA.GFP occurred essentially with efficiencies comparable to those by Nef.GFP. In contrast, the analogous analysis for  $\beta$ Pix, Vav2, and tyrosine-phosphorylated, active Vav (pVav) revealed the presence of a minor fraction of these proteins in DRMs already in the presence of GFP. This residual DRM association remained unaltered by coexpression of Nef.GFP or its AxxA mutant. The distribution of DOCK2/ELMO1 was analyzed upon coexpression of both units of the bipartite GEF and revealed its virtual absence from DRMs irrespective of whether GFP or Nef.GFP was coexpressed. Together, these results were compatible with potential roles of Vav1 and/or  $\beta$ Pix in the Nef-PAK2 complex but did not support an involvement of DOCK2/ELMO1.

#### Vav1 is targeted to plasma membrane microdomains by Nef.

To verify the above results by an independent experimental approach, plasma membrane microdomains were visualized in parallel by confocal microscopy via clustering of fluorescently labeled CTx (24, 34) (Fig. 2). While Vav1 was found diffusely distributed in the cytoplasm of GFP-expressing JTag T lymphocytes (Fig. 2A), the presence of Nef.GFP and AxxA.GFP

led to a marked redistribution of Vav1 to the plasma membrane. Both Nef.GFP variants localized to punctuate structures at the plasma membrane as well as intracellular perinuclear membranes that were detectable in only some z-sections of the cells and likely represent endosomal compartments (27, 41, 71). The Nef-positive plasma membrane punctae partially colocalized with CTx clusters, identifying them as membrane microdomains. In typically two to three of these clusters per optical section, Nef.GFP and Vav1 were found to colocalize. Thus, Nef triggers plasma membrane recruitment of Vav1 and leads to some incorporation of the GEF in plasma membrane microdomains. Coexpression of  $\beta$ Pix did not have appreciable effects on the localization of Nef.GFP (Fig. 2B). Likewise, this GEF was found in the cytoplasm as well as at the plasma membrane, where it sometimes accumulated at CTx-positive clusters. This localization remained unaltered in the presence of Nef, and both proteins were occasionally found to colocalize in membrane microdomains, albeit less frequently than Nef and Vav1. Finally, DOCK2 (Fig. 2C) and ELMO1 (Fig. 2D) were found diffusely in the cytoplasm without marked plasma membrane enrichment or microdomain localization irrespec-



**FIG. 3.** Effect of RNAi knockdown of PAK1 and PAK2 on Nef-associated PAK activity. (A) JTag T lymphocytes were transfected with small interfering RNA oligonucleotides specific for PAK1 or PAK2 or a nonspecific control oligonucleotide (con) and analyzed by Western blotting for PAK1 and PAK2 expression levels at 48 and 72 h post-transfection, respectively. 14-3-3 was used as a loading control. (B) Analysis of Nef-associated PAK activity following PAK1 and PAK2 knockdown. JTag T lymphocytes were transfected with small interfering RNA oligonucleotides specific for PAK1 or PAK2 or the control oligonucleotide together with an expression plasmid for Nef.GFP or the AxxA.GFP mutant, and an IVKA following anti-GFP immunoprecipitation (IP) was performed at 48 (PAK1) or 72 (PAK2) hours posttransfection. Nef-associated PAK activity is revealed by the phosphorylated 62-kDa band (IVKA, pPAK2). (C) Quantification of the Nef-associated PAK activity. Intensities of autophosphorylated PAK2 signals were quantified relative to the amounts of immunoprecipitated Nef.GFP. The relative associated PAK activity for Nef.GFP in the presence of a control oligonucleotide was arbitrarily set to 100%. Data are means  $\pm$  standard deviations from at least five independent experiments. Statistical significance is indicated by the *P* values derived from Student's *t* test analysis. wt, wild type.

tive of coexpression of GFP or Nef.GFP. This independent analysis therefore confirmed a possible role of Vav1 and  $\beta$ Pix in signaling events initiated by Nef from membrane microdomains.

**Nef-associated kinase activity following PAK knockdown.** We next sought to address the role of the three GEFs more directly using RNAi-mediated reduction of protein levels. To this end, we first estimated the range of inhibition of PAK enzymatic activity that can be achieved by RNAi targeted against the kinase itself. Furthermore, while PAK2 has been identified as the preferred PAK isoform associated with Nef, some reports also suggested the involvement of PAK1 (19, 46). We therefore compared the effects of RNAi against these two kinases on Nef-associated PAK activity. As shown in Fig. 3A, RNAi oligonucleotides directed against target sequences in

PAK1 or PAK2, respectively, effectively and specifically reduced the protein levels of the respective PAKs (knockdown efficiency as quantified by Western blotting: PAK1, 88%  $\pm$  15%; PAK2, 94%  $\pm$  10%). Following immunoprecipitation of various Nef.GFP fusion proteins and subsequent IVKA reaction, Nef-associated phosphorylated PAK2 was visualized by autoradiography (Fig. 3B). When an irrelevant RNAi oligonucleotide was used, Nef.GFP associated with robust PAK activity resulting in PAK autophosphorylation (62 kDa, pPAK2) as well as phosphorylation of an 80-kDa substrate. As expected, AxxA.GFP failed to associate with detectable kinase activity. The substantial reduction of PAK1 expression did not result in diminished Nef-associated PAK activity, whereas treatment with the PAK2-specific RNAi oligonucleotide caused about 35% inhibition of Nef-associated kinase activity. When the Nef-associated PAK activity from five experiments was quantified by phosphorimager analysis relative to the amounts of immunoprecipitated Nef.GFP, PAK2 RNAi reduced the kinase reaction to 65%  $\pm$  19% (*P* = 0.001), while PAK1 RNAi had no effect. Consistently, PAK2 but not PAK1 could be detected in anti-Nef immunoprecipitates by using highly sensitive luminometric detection of PAK-luciferase fusion proteins (data not shown). These results suggest that PAK1 does not contribute to Nef-associated kinase activity in our experimental system and indicate that PAK2 protein still present in the RNAi-targeted cells (approximately 6% of the levels in control cells) can be efficiently recruited by Nef to give rise to a relatively high (65% of control) residual Nef-associated kinase activity.

**RNAi knockdown of Nef-associated GEFs.** Having determined the degree of inhibition of Nef-associated PAK2 activity that can be achieved by direct RNAi knockdown of PAK2 itself, we next tested the effect of knockdown for the GEFs previously implicated in Nef-associated protein assemblies. RNAi against Vav1, Vav2,  $\beta$ Pix, and ELMO1 caused a marked reduction in the expression levels of the corresponding endogenous proteins (knockdown efficiency as quantified by Western blotting: Vav1, 86%  $\pm$  15%; Vav2, 76%  $\pm$  10%; DOCK2, 97%  $\pm$  5%; ELMO, 85%  $\pm$  9%;  $\beta$ Pix, 86%  $\pm$  10%). Potent silencing of DOCK2 protein expression was confirmed using a transfected vector expressing an epitope-tagged version of DOCK2 (Fig. 4A). IVKA analysis revealed a significant reduction of Nef-associated PAK2 activity upon Vav1 RNAi treatment. The magnitude of this effect was comparable to that obtained with direct knockdown of PAK2 (reduction to 68%  $\pm$  20% for Vav1 and 65%  $\pm$  19% for PAK2). RNAi against Vav2 and ELMO1 (reduction to 71%  $\pm$  20% and 67%  $\pm$  15%, respectively) also had statistically significant effects on the Nef-PAK2 association (Fig. 4B and C; *P* values indicate statistical significance in comparison to reaction after treatment with RNAi control oligonucleotides). Knockdown of DOCK2 and  $\beta$ Pix caused no significant alterations in Nef-associated PAK2 activity (reduction to 89%  $\pm$  20% and 88%  $\pm$  25%). These results further suggested a critical role for Vav1 in the Nef-PAK2 complex and argued against the involvement of DOCK2 and  $\beta$ Pix.

**Impairment of Nef-PAK2 association in Vav1-deficient T lymphocytes.** Because inhibition of Nef-associated PAK2 activity was only partial upon RNAi-mediated reduction of Vav protein levels, we next turned to the JVav cell line, a Jurkat T-lymphocyte derivative that lacks Vav1 expression due to

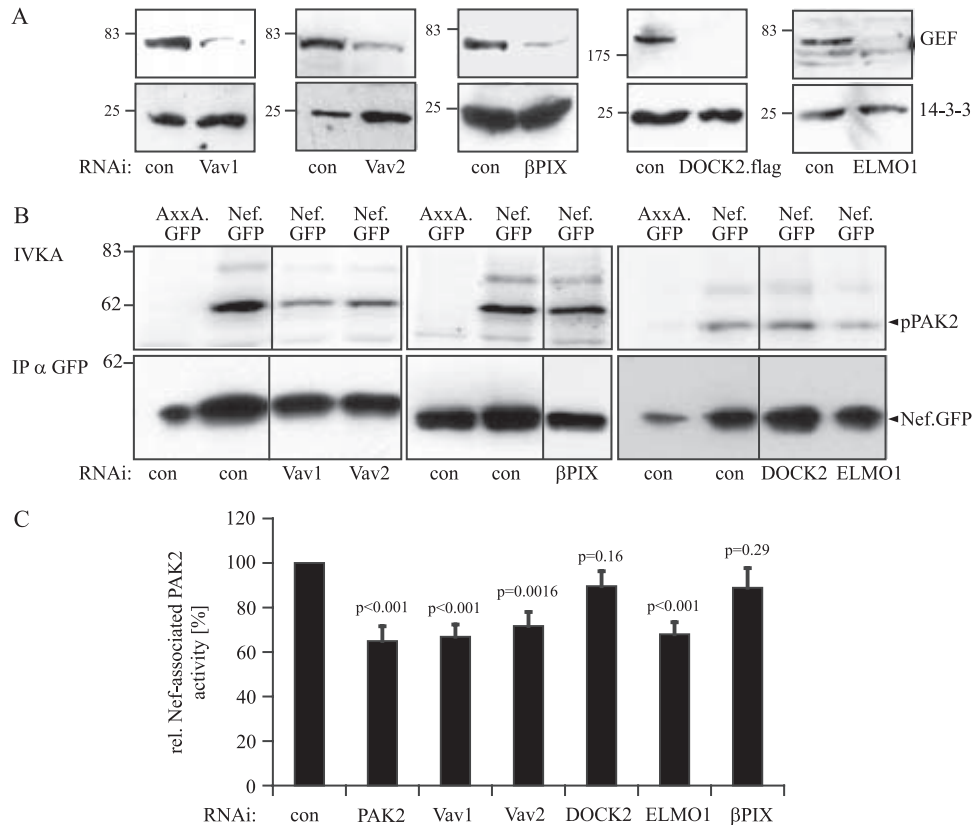


FIG. 4. Effect of RNAi knockdown of cellular GEFs on Nef-associated PAK2 activity. (A) Western blot analysis of JTag cells transfected with small interfering RNA oligonucleotides specific for the indicated GEFs or a nonspecific control oligonucleotide (con) for GEF expression levels, as in Fig. 3. Note that endogenous protein levels were analyzed, except in the case of DOCK2, where an expression plasmid for DOCK2.flag was cotransfected with the RNAi oligonucleotides. (B) Nef-associated PAK2 activity following GEF knockdown. JTag T lymphocytes transfected with GEF-specific small interfering RNA or the control oligonucleotide together with the indicated Nef.GFP expression plasmids were subjected to IVKA following anti-GFP immunoprecipitation (IP). (C) Quantification of the Nef-associated PAK2 activity following GEF knockdown. Intensities of autophosphorylated PAK2 signals were quantified relative to the amounts of immunoprecipitated Nef.GFP. The relative associated PAK2 activity for Nef.GFP was arbitrarily set to 100%. Data are means  $\pm$  standard errors of the means from at least five independent experiments. Statistical significance is indicated by the *P* values derived from Student's *t* test analysis. wt, wild type.

genetic knockout (12). Consistent with the previous RNAi experiments, the Nef protein from HIV-1 SF2 associated up to fourfold less efficiently with PAK2 activity in the absence than in the presence of Vav1 (Fig. 5A, compare JVav with JTag). Although with various degree of reduction in Nef-associated PAK2 activity in the absence of Vav1, the involvement of Vav1 in Nef-PAK2 association was also conserved for SIVmac239 Nef, the T71R mutant of Nef from HIV-1 NL4-3, and the patient derived HIV-1 Nef variant RP4-11 (Fig. 5B). To verify that this reduction in Nef-associated PAK2 activity was really due to the lack of Vav1, a myc-tagged version of Vav1 was coexpressed with Nef in JVav cells (Fig. 5C). Under conditions where expression levels of overexpressed Vav1.myc were in the range of endogenous Vav1 in JTag cells, Nef-PAK2 association was markedly enhanced, reaching levels that almost matched those in parental JTag cells. Since Nef can associate with Vav2 as well as with Vav1 (20), we next tested whether the residual Nef-PAK2 association in JVav cells was due to the presence of Vav2 and performed RNAi knockdown experiments targeting Vav2 (Fig. 5D). As seen before in JTag cells, Vav2 knockdown was only partial in JVav cells; however, it reduced the residual Nef-associated PAK2 activity (to  $54\% \pm 15\%$  and  $41\% \pm 20\%$

for HIV-1 SF2 and SIVmac239 Nef, respectively). Together these results support an important role of Vav1 in the Nef-PAK2 complex and suggest that Vav2 can substitute for the Vav1 isoform.

**DOCK2 expression and  $\beta$ Pix binding of PAK2 are dispensable for the association of Nef with PAK2 activity.** Following the genetic knockout approach, the role of DOCK2 in the association of Nef with PAK2 activity was tested using DOCK2<sup>-/-</sup> BE $\alpha$ 16-3 T-cell hybridoma cells (58). No significant difference was observed in the PAK2 association of Nef proteins from HIV-1 and SIV between BE $\alpha$ 16-3 and JTag cells (Fig. 6A), strongly suggesting that DOCK2 is dispensable for Nef-PAK2 association. Of note, reduction of Vav1 expression using RNAi in BE $\alpha$ 16-3 cells caused a twofold reduction of Nef-PAK2 association (Fig. 6B). Experiments on a  $\beta$ Pix<sup>-/-</sup> genetic background were precluded due to the lack of a  $\beta$ Pix knockout cell line. Since  $\beta$ Pix directly binds to PAK2 (29), we tested instead whether a PAK2 mutant carrying a mutation in the  $\beta$ Pix binding site would be able to associate with Nef in its catalytically active form. As presented in Fig. 6C, association with PAK2 activity was equally efficient for HIV-1 as well as SIV Nef in the presence of wt and  $\beta$ Pix binding-deficient

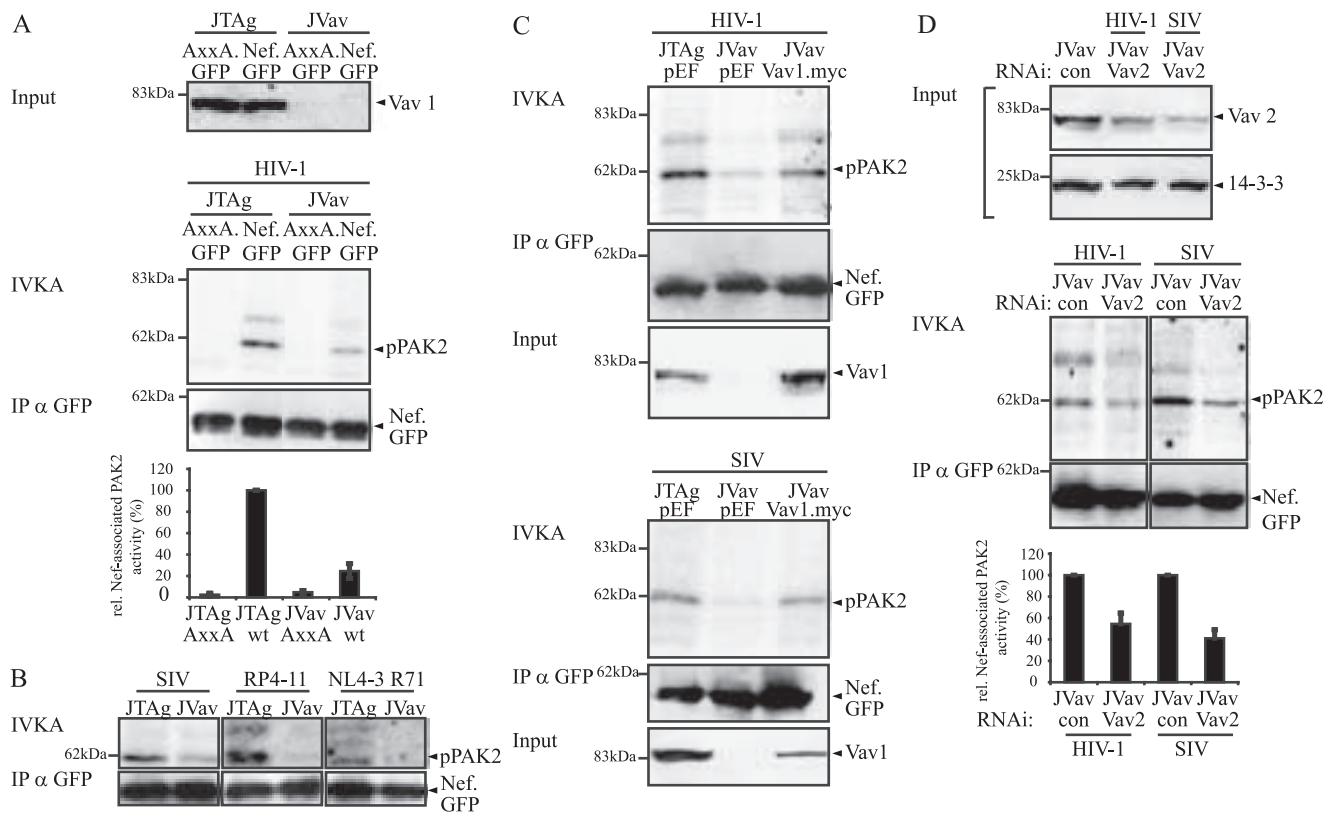


FIG. 5. Vav1 deficiency reduces Nef-PAK2 association in T lymphocytes. (A) IVKA for Nef-associated PAK2 activity in JTag and Vav1-deficient JVav T lymphocytes. IVKA reactions were performed for Nef from HIV-1 SF2. Vav1 expression levels are shown in the upper panels; the middle panel depicts PAK2 autophosphorylation levels and amounts of immunoprecipitated Nef. The graphs present relative levels of Nef-associated PAK2 activity, with values for wild-type (wt) Nef in JTag cells set to 100%. Data are means  $\pm$  standard errors of the means from three independent experiments. IP, immunoprecipitation. (B) IVKA reaction and Western blot analysis for immunoprecipitated Nef from SIVmac239, HIV-1 RP4-11, and HIV-1 NL4-3. T71R, HIV-1 RP4-11, and SIVmac239. (C) Rescue of HIV-1 SF2 Nef-associated PAK2 activity in JVav cells by Vav1 overexpression. Shown are the IVKA reaction, amounts of immunoprecipitated Nef, and Vav1 expression levels. (D) Residual Nef-associated PAK2 activity in JVav cells is sensitive to RNAi against Vav2. JVav cells were treated with the indicated RNAi oligonucleotides, and knockdown efficiency was evaluated by Western blotting (upper panel). From the same lysates, an IVKA was performed (middle panel). The bottom panel presents the quantification of Nef-associated PAK2 activity, with values for wt Nef in JVav cells treated with a control oligonucleotide set to 100%. Data are means  $\pm$  standard errors of the means from three independent experiments.

PAK2 variants. In conjunction with the RNAi experiments, these results suggested that  $\beta$ Pix is dispensable for the association of Nef with active PAK2. Taking together analyses on GEF membrane microdomain recruitment, GEF RNAi, and the use of GEF knockout cell lines, we conclude that among the three GEFs previously associated with Nef function, only Vav1 fulfils the criteria for a GEF with a major role in the Nef-PAK2 complex.

**Residue 195 is critical for the Nef-PAK2 association and Vav1 recruitment.** The identification of the SH3 domain-containing Vav1 protein as the relevant GEF in the Nef-PAK2 complex was at odds with the fact that its recruitment into membrane microdomains occurred independently of the highly conserved SH3 binding (PxxP) motif of Nef. Recent studies identified a novel protein interaction surface involving residue F195 in Nef that is required for the Nef-PAK2 association (2, 49). We therefore tested whether this surface is involved in the recruitment of Vav1 by Nef and created expression vectors for F195A and F195I Nef.GFP proteins. Compared to wt Nef, both mutants displayed less than 5% of Nef-associated PAK2 activity and scored lower than the AxxA.GFP or the membrane binding-deficient

G2A.GFP controls (Fig. 7). DRM flotation experiments and confocal microscopy revealed that this lack of kinase association was not a consequence of exclusion of the F195 mutants from membrane microdomains (Fig. 8A) or other mislocalization in T lymphocytes (Fig. 8B). Rather, the association with DRM fractions appeared to be slightly elevated relative to Nef.GFP. In sharp contrast, both Nef variants with mutations at position F195 failed to recruit Vav1 into DRMs. Consistently, F195A.GFP (Fig. 8B) and F195I.GFP (data not shown) were defective in targeting Vav1 to the T-lymphocyte plasma membrane and did not induce colocalization of the GEF with CTx membrane microdomains. We conclude that the F195 protein interaction surface is a critical determinant for the membrane microdomain recruitment of Vav1 and thus for the association of Nef with PAK2 activity.

## DISCUSSION

The composition of the Nef-PAK2 complex has remained controversial largely due to its low stability and the concomitant difficulties in detecting associated proteins directly rather than based on their activity. In a first step toward understand-



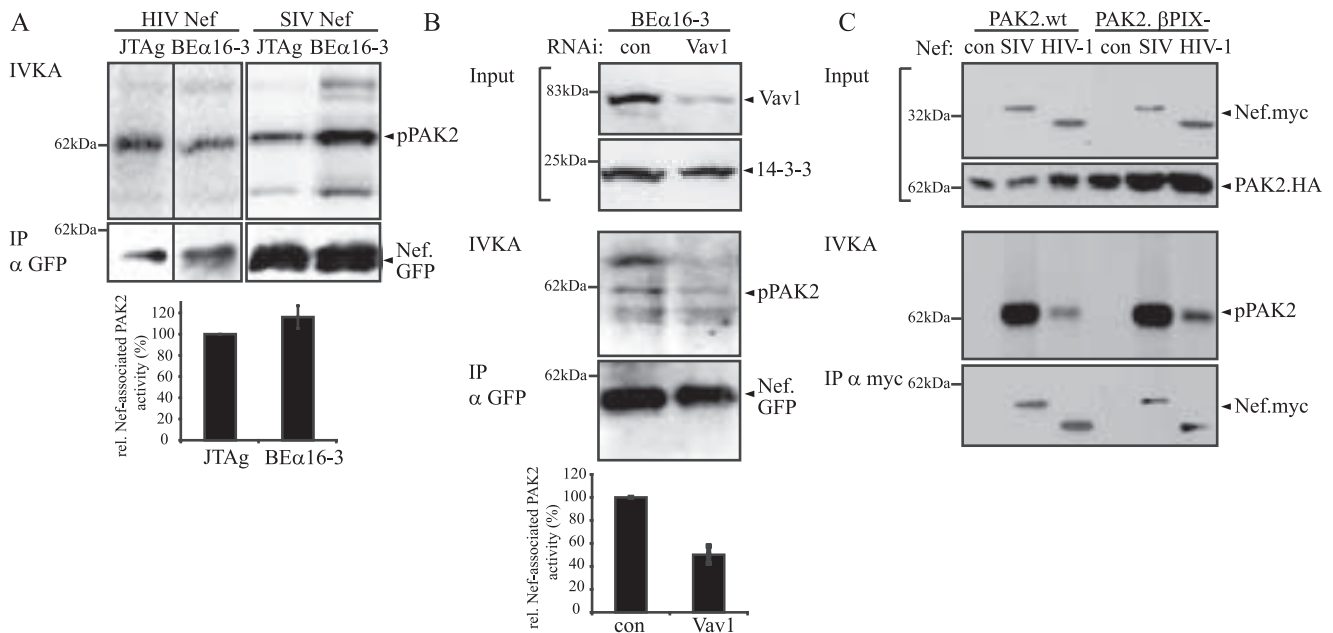


FIG. 6. Nef-PAK2 association is functional in the absence of DOCK2 or the presence of βPix binding-deficient PAK2. (A) IVKA for Nef-associated PAK2 activity in JTag and DOCK2-deficient BEα16-3 cells. The IVKA reaction for Nef from HIV-1 SF2 and SIVmac239 and amounts of immunoprecipitated Nef are shown in the upper panels; the lower panel depicts relative levels of HIV-1 and SIV Nef-associated PAK2 activity, with values for wild-type (wt) Nef in JTag cells set to 100%. Data are means ± standard errors of the means from four independent experiments. IP, immunoprecipitation. (B) Nef-associated PAK2 activity is reduced upon Vav1 knockdown. BEα16-3 cells were treated with the indicated RNAi oligonucleotides. Knockdown efficiency was evaluated by Western blotting (upper panels). IVKA analysis and quantification of amounts of immunoprecipitated Nef.GFP are presented in the middle panels. The bottom panel presents the quantification of Nef-associated PAK2 activity, with values for Nef in BEα16-3 cells treated with a control oligonucleotide set to 100%. Data are means ± standard errors of the means from three independent experiments. (C) βPix binding is dispensable for functional Nef-PAK2 association. PAK2 association was analyzed for Nef from HIV-1 SF2 and SIVmac239 in the presence of wt or βPix binding-deficient PAK2. From top to bottom, the panels show expression levels of Nef and PAK2, the IVKA reaction, and the amounts of immunoprecipitated Nef.

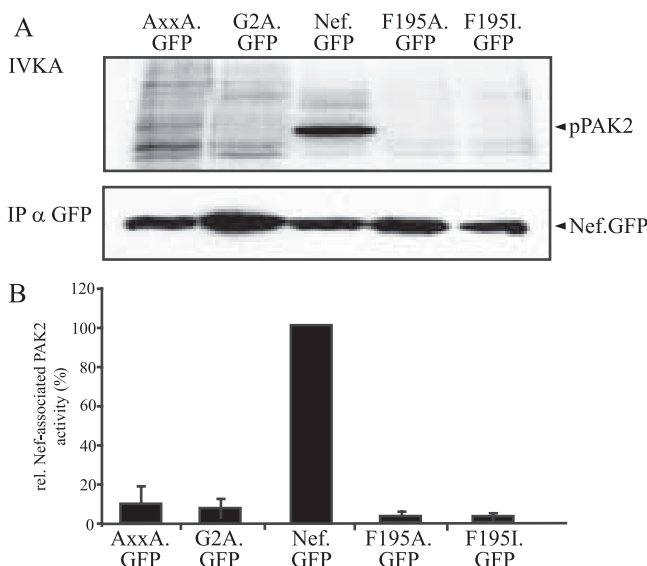


FIG. 7. F195 is critical for the association of Nef.GFP with PAK2 activity. (A) IVKA analysis from JTag T lymphocytes transiently expressing the indicated Nef.GFP fusion proteins. IP, immunoprecipitation. (B) Quantification of PAK2 activity associated with the Nef.GFP variants analyzed in panel A. The relative associated PAK2 activity for Nef.GFP was arbitrarily set to 100%. Data are means ± standard deviations from at least three independent experiments. wt, wild type.

ing the organization and regulation of this signalosome, we addressed here which of the reported Nef-associated cellular GEFs plays a role in the association of Nef with PAK2 activity. Microdomain recruitment, subcellular localization, GEF-specific RNAi, genetic knockout cells, and GEF binding-deficient PAK2 variants were applied as independent assays and tools to answer this question. Taken together, the results reveal a role for Vav1 in the association of Nef with PAK2 activity and argue that βPix and DOCK2 are dispensable for this Nef function. Surprisingly however, a recently described protein interface surrounding F195 rather than the SH3 binding PxxPxR motif in Nef mediates the recruitment of Vav1 into the signalosome.

Since each individual experimental strategy used here to dissect the role of cellular GEFs in functional Nef-PAK2 association had its specific limitations, it is important to consider the combined interpretation of the independently obtained results. The isolation of DRMs demonstrated Nef-mediated recruitment of Vav1 but not DOCK2/ELMO1, while βPix was found to be DRM resident with no detectable alteration upon Nef expression. Due to possible artifacts during detergent treatment, these results were compared with microdomain cluster colocalization. Besides providing overall confirmation of the biochemical analyses, this approach also allowed us to appreciate the specific recruitment of Vav1 by Nef to the plasma membrane on a single-cell level, which was overall more pronounced than the microdomain targeting judged by

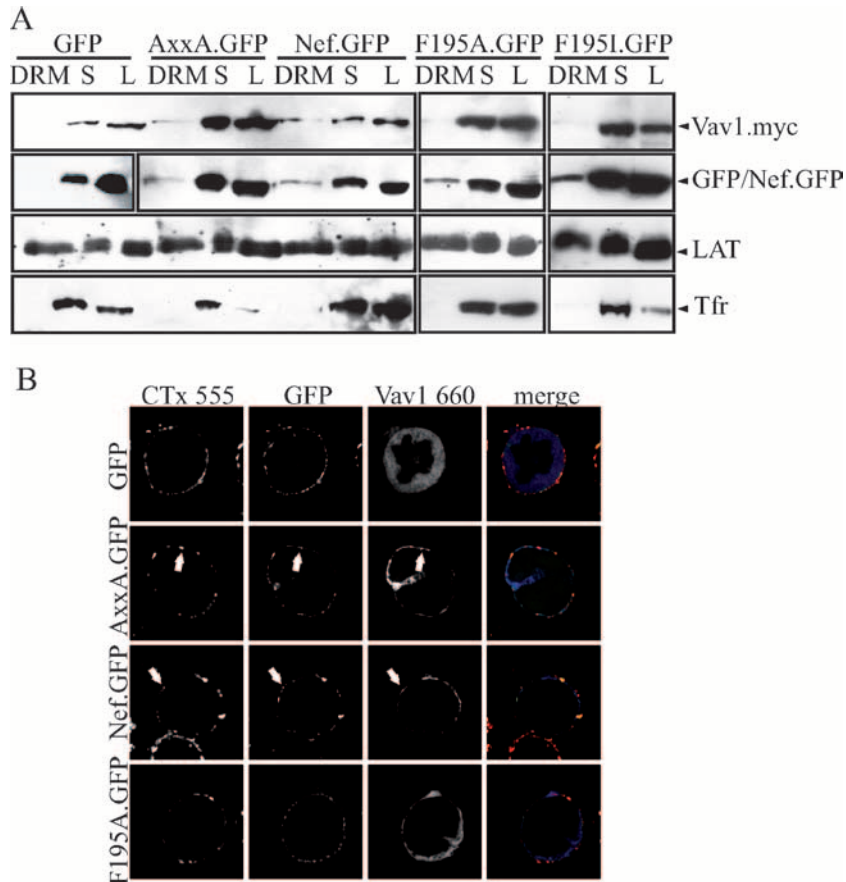


FIG. 8. Role of F195 in microdomain recruitment of Vav by Nef. (A) Western blot analysis of DRM flotation from JTag T lymphocytes transiently expressing the indicated Nef.GFP fusion proteins or GFP together with myc-tagged Vav1 (see legend to Fig. 1 for details). (B) Raft clustering analysis of the JTag T lymphocytes analyzed in panel A. Cells were analyzed by confocal microscopy, and single representative sections are presented (see legend to Fig. 2 for details). The merge panel depicts the overlay of all three fluorescence channels, with GFP/Nef.GFP in green, CTx in red, and Vav1 in blue. Arrows indicate colocalization of Nef with Vav1 in plasma membrane microdomains. The results presented are representative of at least three independent experiments.

biochemical criteria. The lack of detection of DOCK2/ELMO1 in DRMs or CTx clusters in Nef-expressing cells argued against a functional role of this bipartite GEF for the Nef-PAK2 association; however, the catalytic relevance of undetectable protein amounts could not be excluded. However, since the recruitment of other catalytic components of the Nef-PAK2 complex, such as PAK2, Rac1, and Cdc42, can readily be demonstrated (34), a critical involvement of DOCK2/ELMO1 seemed unlikely. The microdomain analysis also did not yield information on the functional relevance of membrane microdomain-associated GEFs and could thus not differentiate between the roles of Vav and  $\beta$ Pix. GEF-specific RNAi was therefore used in further analyses. Knockdown of PAK1 and PAK2 established the dynamic range that could be expected for this approach: despite reduction of PAK2 expression to less than 10% of the levels in control cells, only an approximately 30% decrease in Nef-associated PAK activity was observed. Similar difficulties in functional knockdowns of cellular enzymes, including PAK2, have been encountered by other investigators (37–39, 73) and possibly reflect cells' ability to maintain a relatively constant pool of active enzymes even upon significant reduction of overall enzyme abundance. Al-

ternatively, and not mutually exclusive, these results could also indicate the presence of a PAK2 phosphorylating activity in the complex other than just PAK2 itself. These findings are in agreement with the finding that Nef specifically associates with a small but highly active PAK2 subpopulation (51, 55). Providing specificity to these results, similarly efficient knockdown of PAK1 expression did not affect Nef-associated PAK activity. In a previous study we had concluded that PAK1 can serve as a Nef-associated kinase based on the inhibitory effect of an interfering PAK1 fragment (19). This fragment was, however, subsequently shown to also interfere with the activity of PAK2. Use of specific antibodies and sensitivity to caspase cleavage then identified PAK2 as the Nef-associated kinase (5, 54). The RNAi data presented here confirm that PAK2 is the relevant Nef-associated PAK isoform in JTag T lymphocytes by an independent experimental approach. Within the limits of this incomplete inhibition of Nef-associated PAK2 activity, efficient knockdown of  $\beta$ Pix and DOCK2 had no significant effect on Nef's association with PAK2 activity. Consistently, binding of  $\beta$ Pix to PAK2 was fully dispensable for efficient association of Nef with PAK2 activity. More directly, BE $\alpha$ 16-3 cells allowed us to verify the involvement of DOCK2 in a cellular environ-

ment of genetic DOCK2 deficiency. The results clearly demonstrated that DOCK2 expression is dispensable for the association of Nef with PAK2 activity. Thus,  $\beta$ Pix and DOCK2 could be excluded as constituting limiting factors in the Nef-PAK2 complex. In contrast, Vav1, and to a lesser extent Vav2, were identified as relevant for functional Nef-PAK2 interactions, with the effects of Vav1 knockdown being as pronounced as knockdown of the kinase itself. Analysis of T lymphocytes devoid of Vav1 expression confirmed a critical role of Vav1 in Nef's association with PAK2 activity. Importantly, Nef-PAK2 association was rescued in these cells by reintroduction of Vav1, and residual Nef-PAK2 association was sensitive to expression levels of Vav2. Together with the microdomain analysis, these results demonstrate a functional role for Vav1 and Vav2 but not DOCK2 or  $\beta$ Pix in the Nef-PAK2 complex.

DOCK2/ELMO1 associate with Nef in a PxxP-dependent manner and have been implicated in the inhibition of T-lymphocyte chemotaxis by the viral protein (28). This effect likely depends on the exchange activity of this GEF for Rac1, which is exerted only in the context of a direct interaction of both subunits of this bipartite GEF (10, 58). Since DOCK2 was found to be entirely dispensable for the association of Nef with active PAK2, this GEF appears not be involved in this function of Nef. In line with these findings, Janardhan and colleagues did not detect PAK2 or Vav1 proteins in their Nef-associated DOCK2-ELMO1-Rac complex (28). Together, these results suggest that the association of Nef with PAK2 and with DOCK2/ELMO1 occurs in the context of two independent protein complexes. Thus, Nef, via its PxxPxR motif, assembles multiple protein complexes in T lymphocytes that differ in abundance, composition, and possibly subcellular localization to modulate select effector functions. In contrast to DOCK2, however, knockdown of ELMO1 partially affected the functional Nef-PAK2 association, suggesting that ELMO1 can affect Nef-PAK2 independently of DOCK2. As ELMO1 was undetectable in Nef-positive microdomains, we favor the idea that these effects are indirect consequences of reduced ELMO1 expression levels, possibly reflecting the regulation of Rac signaling pathways by ELMO1 in conjunction with DOCK family members other than DOCK2 (26).

Regarding the role of  $\beta$ Pix in the Nef-PAK2 complex, it was previously reported that this GEF is physically present in the complex (75) and serves as a substrate of Nef-associated active PAK2 (9). On the other hand, we show here that the  $\beta$ Pix-PAK2 interaction is dispensable for Nef's ability to associate with active PAK2. Together these results suggest that  $\beta$ Pix, possibly facilitated by its constitutive microdomain association and recruited via its interaction with PAK2, associates with the Nef-PAK2 complex as a peripheral complex component which does not contribute to PAK2 activity levels in this context. Rather,  $\beta$ Pix might act as a downstream effector of Nef-PAK2 signalosomes. In line with such a model,  $\beta$ Pix was recently shown to associate with Nef in membrane microdomains to regulate the activity of the ubiquitin ligase c-Cbl (67).

Vav1 is the only GEF tested here that was recruited to membrane microdomains by Nef and whose expression levels directly correlated with the efficiency of the association of Nef with PAK2 activity. These results thus identify endogenously expressed Vav1 as a critical component for the

function of the Nef-PAK2 complex, thereby confirming and extending previous reports on the interaction of Nef with this particular GEF (20, 52, 74). Although not experimentally addressed here, this role most likely involves its GEF activity toward Rac1 and Cdc42, which, in their GTP bound state, ensure activity of PAK2 in the complex prior to its disassembly. Nef was previously shown to interact directly with the C-terminal SH3 domain of Vav1 *in vitro* via its PxxPxR motif, and the association of Nef and Vav1 in cells was also shown to depend on these interaction surfaces (20, 52, 74). These results suggested that Vav1 might be recruited into the Nef-PAK2 complex via this interaction. Unexpectedly, however, we find here that this recruitment occurs via the F195 patch rather than the Nef SH3 binding motif. In addition to Vav1, the association of PAK2 with Nef also depends on the F195 interaction surface (2). These data do not allow us to identify the direct interaction partner of this Nef interface, which could represent either one of the two proteins or a yet-unidentified adapter protein. The results presented also raise the question of which SH3 protein interacts with Nef's PxxPxR motif to facilitate functional Nef-PAK2 interactions. While the C-terminal SH3 domain of Vav1 might indeed interact with this interface within the complex, our data argue that this interaction would not significantly contribute to the stability of the Nef/Vav1/PAK2-containing signalosome. In this scenario, the PxxP-SH3 interaction might ensure correct positioning of the complex and/or Vav activity and thus provide activation instead of recruitment of critical complex components. Affinity screening of a library containing the complete collection of human SH3 domains did not reveal any SH3 domains expressed in T cells that would show distinct affinity for Nef (29), thus indirectly supporting the idea that the critical function of the Nef PxxP motif in Nef/Vav1/PAK2 assembly might be mediated via such a low-affinity SH3 interaction. Alternatively, the activity of the complex would also be facilitated by the association with yet-to-be-identified SH3 domain-containing factors that may well lack GEF activity.

The association of Nef with PAK2 activity was among the first protein interactions described for this viral pathogenicity factor (59) and represents one of the most conserved features of the different HIV and SIV Nef variants (33). However, due to the low stability of the Nef-PAK2 complex and the involvement of protein interaction surfaces required for complex assembly in several other independent Nef activities, the physiological role and the molecular principles of this complex have remained largely elusive. Based on the new insights into the Nef-PAK2 complex organization presented here, future studies can now focus on determining the full composition of the Nef-PAK2 complex and on the specificity of its downstream effector functions.

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