STRUCTURAL AND FUNCTIONAL ANALYSIS OF HIV-1 NEF
ACTIVATION OF PAK-2

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DEDICATION

This is dedicated to my parents and sister.

STRUCTURAL AND FUNCTIONAL ANALYSIS OF HIV-1 NEF ACTIVATION OF PAK-2

by

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DISSERTATION

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF HIV-1 NEF ACTIVATION OF PAK-2

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Nef is an accessory protein encoded by HIV-1 that activates the host cellular p21 activated protein kinase 2 (PAK-2). Previous work has characterized the structural plasticity of Nef with regard to PAK-2 activation. Residues 89 and 191 were identified to be components of an effector domain required for Nef mediating PAK-2 activation with lesser contributions from position 85 and 188. H89 and F191 are highly conserved in subtype B Nefs (LHKF), however in subtype E Nef F89 and R191 predominate. Subtype E Nefs also activate PAK-2, therefore it appeared at least two different structural variants are present in HIV-1 Nefs. Substitution of all four residues in a subtype B Nef with subtype E-like residues (F85, F89, A188 and R191,

FFAR) generated a fully functional subtype E PAK-2 effector domain in a subtype B background.

A third effector domain found in subtype C Nefs (F85, F89, H188, and H191, FFHH) was also investigated. The contribution of residues 187 and 188 in these alternative Nef structural variants (LHKF, FFAR, and FFHH) to activate PAK-2 was determined. Surprisingly, the L188 substitution in the LHKF structure resulted in PAK-2 hyperactivation. While the I187 substitution in LHKF completely ablated PAK-2 activity. In stark contrast, I187 in the FFHH variant resulted in hyperactivation. Thus, subtle changes in amino acid composition can dramatically affect kinase activation levels.

The work in this thesis has characterized a PAK-2 effector domain on Nef constituted by amino acid position 85, 89, 187, 188 and 191. The results indicate that this is not the only Nef region mediating PAK-2 activation. The highly conserved polyproline helix also plays a role in the activation of PAK-2. Conservative mutations of this SH3 binding region completely abrogated PAK-2 activation suggesting SH3 binding is necessary, however this binding appears to be weak. My data suggest a model where activation of PAK-2 by Nef requires a ternary, or higher order, complex containing SH3/Nef/PAK-2. Synergistic interactions between the two Nef effector domains investigated here and a host cell protein, or proteins, could explain the specific activation of PAK-2 by Nef.

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LIST OF DEFINITIONS

AIDS	Acquired Immunodeficiency Syndrome
AP	adaptor protein
APOBEC3G	apolipoprotein B mRNA editing enzyme 3G
Arf	ADP ribosylation factor
ARNO	Arf nucleotide binding site opener
CA	capsid
CD4	cluster of differentiation 4
cDNA	complementary DNA
CK2	casein kinase 2
CTL	cytotoxic T lymphocyte
CTx	cholera toxin
DC	District of Columbia
DNA	deoxyribonucleic acid
Env	envelope
ER	endoplasmic reticulum
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
Gag	group specific antigen
gp	glycoprotein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HIV	Human Immunodeficiency Virus
HTLV	human T-cell lymphotropic virus
IN	integrase
kb	kilobase
kDa	kilodalton
LAV	lymphoadenopathy-associated virus
LTR	long terminal repeat
MA	matrix
MAPK	mitogen activated protein kinase
MHC-I	major histocompatibility complex I
mRNA	messenger RNA
NC	nucleocapsid
Nef	negative factor
Nf2	neurofibromatosis
NIH	National Institutes of Health

NMR	nuclear magnetic resonance
ORF	open reading frame
PACS-1	phosphofurin acidic cluster sorting 1
PAK-2	p21 activated kinase 2
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PI3K	phosphoinositide 3 kinase
PIC	pre-integration complex
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristate acetate
Pol	polymerase
PR	protease
Rev	regulator of virion gene expression
RNA	ribonucleic acid
RRE	Rev response element
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	src family kinase
SH3	src homology 3
SIV	simian immunodeficiency virus
TAR	transactivation response element
Tat	trans-activator of transcription
TGN	trans Golgi network
TPA	12-O-tetradeconoylphorbol-13-acetate
UNAIDS	United Nations Programme on HIV/AIDS
UTR	untranslated region
Vpr	viral protein R
Vpu	viral protein U

Chapter 1: Introduction to HIV Biology

HIV is the causative agent of AIDS. Acquired immunodeficiency syndrome (AIDS) was first observed in the early 1980s in patients who presented with opportunistic infections such as Pneumocystis pneumonia and a rare form of cancer called Kaposi's sarcoma, as documented by the CDC in the Morbidity and Mortality Weekly Report (1982). In 1983, two independent groups identified a T-lymphocytic retrovirus to be the causative agent of AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1983). The Gallo group from the National Institutes of Health (NIH) named it human T-cell lymphotropic virus type III (HTLV-III) while the Montagnier group from the Pasteur Institute named it lymphoadenopathy-associated virus (LAV). Further analysis using electron microscopy revealed this retrovirus to be different from HTLVs. The new virus was later renamed human immunodeficiency virus (HIV) (Coffin et al., 1986).

As of 2007, the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated there are approximately 33 million people worldwide living with HIV. As depicted in Figure 1.1, the prevalence of HIV/AIDS is most severe in sub-Saharan Africa. It is estimated that 67% of people in the world living with HIV are in sub-Saharan Africa and this region accounted for 72% of all HIV/AIDS associated deaths in 2007. On average, it is estimated that one out of every twenty

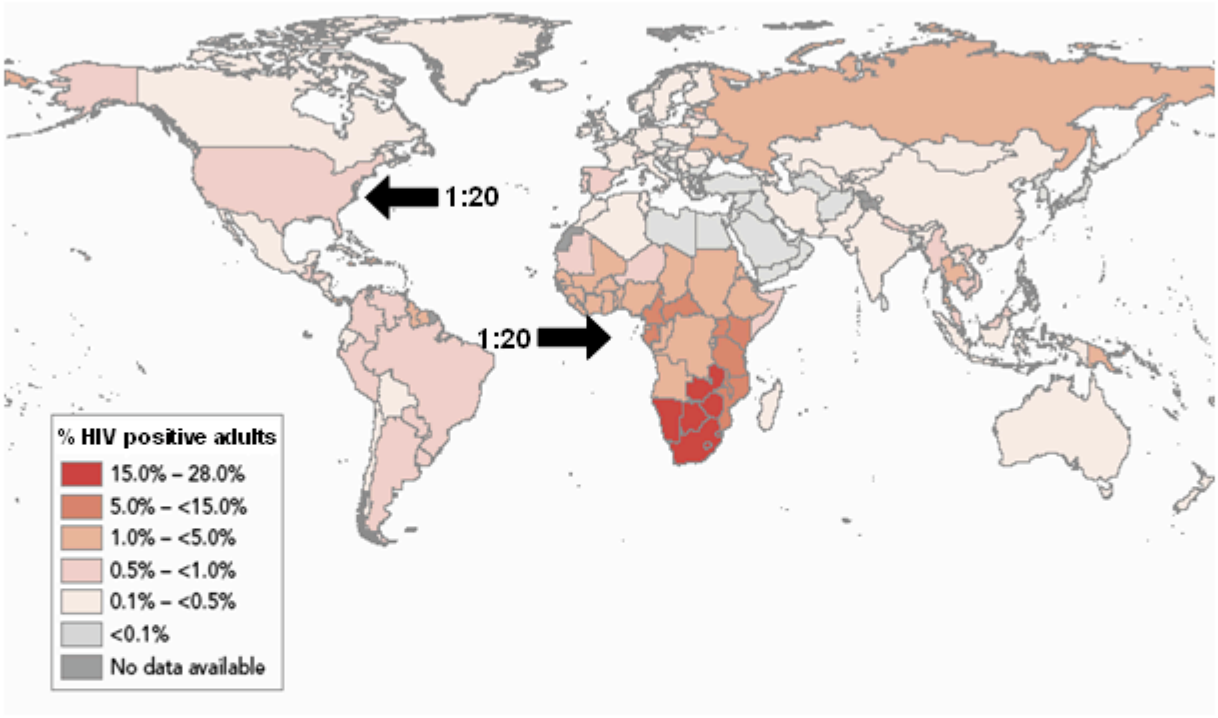

Figure 1.1 The global HIV/AIDS pandemic. As depicted in the darkest red, the pandemic is most severe in sub-Saharan Africa. On average, it is estimated that one in twenty people in sub-Saharan Africa is living with HIV which is equivalent to the rate in the District of Columbia. Figure modified from 2008 UNAIDS Executive Summary (Joint United Nations Programme on HIV/AIDS (UNAIDS) 2008).

people in sub-Saharan Africa is HIV positive. Moreover, it is also estimated that one out of every twenty residents of the District of Columbia (DC) is HIV positive. The incidence of HIV/AIDS in DC is the highest in the United States. The annual rate of new HIV cases in DC is ten times the national average, as described by the DC Appleseed Center (Center, 2005).

HIV-1 subtypes. Primate lentiviruses are a subgenus of retroviruses that include HIV-1, HIV-2, and SIV. HIV-1 is divided into three groups: M, N, and O. HIV-1 group M is subdivided into ten subtypes A-J. Groups N and O are only found in Cameroon while group M is responsible for the worldwide epidemic. All ten subtypes of group M are found in central Africa. Germane to the studies presented here are subtypes B, C, and E, which have spread to disparate regions of the globe, as illustrated in Figure 1.2. Subtype B is found predominantly in North America, Western Europe, and Australia. While subtype C predominates in sub-Saharan Africa and subtype E predominates in southeastern Asia. Group O represents HIV-1 isolates that are highly divergent "Outliers." HIV-1 group N was identified from another divergent isolate, YBF30, that is more closely related to group M than group O HIV-1s (Simon et al., 1998).

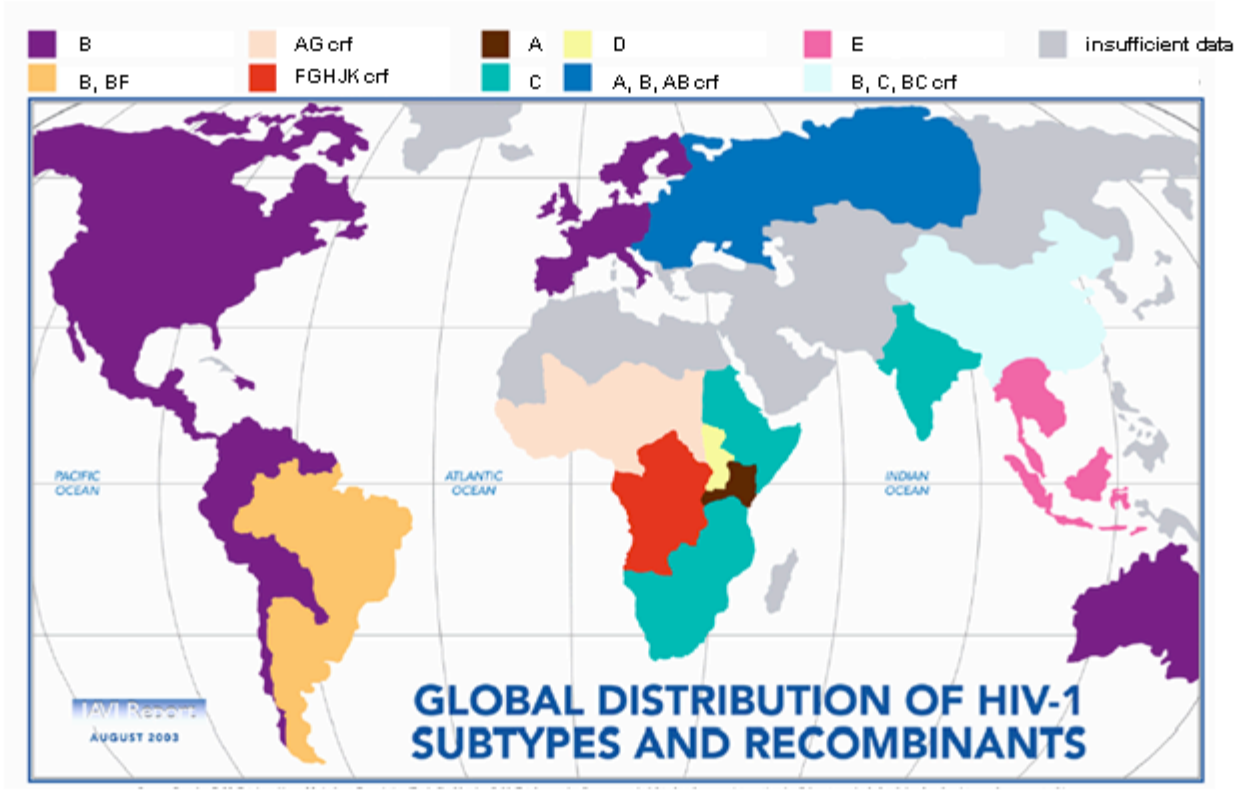

Figure 1.2 The global distribution of HIV-1 subtypes and recombinants. The HIV-1 subtypes most germane to these studies are subtypes B, C and E. Subtype B shown in purple is represented in Europe, North America and parts of South America. Subtype C shown in green is found in sub-saharan Africa and India. Subtype E shown in pink is found in southeast Asia. Figure modified from the International AIDS Vaccine Initiative 2003.

Structural proteins: Gag, Pol, and Env. HIV-1 contains an RNA genome of approximately 9.5 kb packaged in a thimble shaped viral core within a spherically shaped plasma membrane. HIV-1 encodes three structural polyproteins Gag, Pol, and Env, shown in blue in Figure 1.3. Group-specific antigen, or Gag is proteolytically processed by the viral protease (PR) into four proteins: matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (Ganser-Pornillos et al., 2008). MA is a multifunctional protein that coats the inside of the viral membrane and is involved in plasma membrane targeting and viral assembly (Bukrinskaya, 2007). CA proteins assemble into a hexameric units that form the truncated cone of the viral core that encapsidates the viral RNA genome (Briggs et al., 2004). NC is also involved in assembly and packaging of the RNA genome. Finally, p6 in involved in recruiting the host cellular machinery to promote viral budding (von Schwedler et al., 2003).

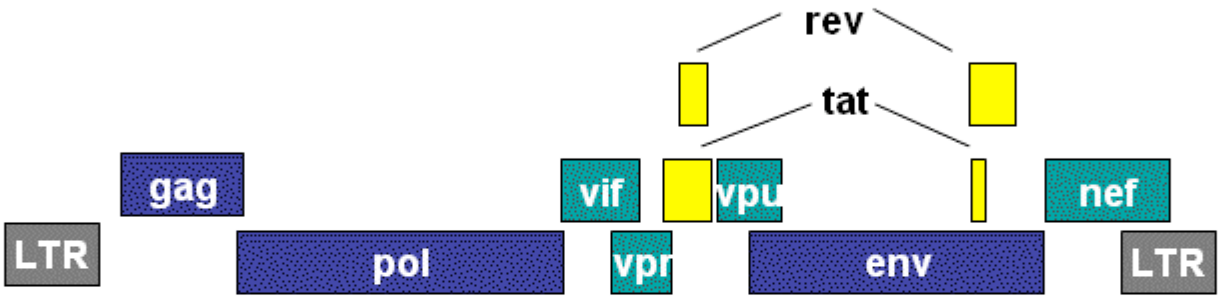

Figure 1.3 Genomic organization of HIV-1. Schematic showing the genomic organization of the nine HIV-1 genes and the long-terminal repeats (LTRs). In blue are the structural genes: gag, pol, and env. In yellow are the regulatory genes: tat and rev. In green are the accessory genes: vif, vpr, vpu and nef.

The *pol* gene protein product, Pol is also cleaved by autocatalysis to yield the three viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). All three of these proteins are included in the HIV-1 virion. PR plays a key role in proteolytically processing the Gag-Pol polyproteins into the various mature viral proteins. PR also cleaves Pol to form the 51 kDa and 66 kDa subunits of the RT dimer. Reverse transcriptase is a misnomer in that RT is not a transcriptase but in fact a RNA or DNA dependent DNA replicase. Upon synthesis of the viral cDNA by RT, IN functions to integrate the viral genome into the cell by covalently inserting the provirus into host DNA (Swanson and Malim, 2008).

The HIV-1 envelope (Env) is an 830-840 amino acid transmembrane spanning protein. Env is first processed by removal of the 20 amino acid signal peptide, thus targeting the Env to the ER lumen where it is heavily glycosylated. Both O-linked and N-liked glycosylation occurs but the bulk of the Env associated carbohydrate is N-linked. The 160 kDa Env glycoprotein is then further processed in the ER lumen by a furin-like protease to generate the gp120 and gp41 cleavage products (Swanson and Malim, 2008). Despite cleavage, gp120 remains noncovalently bound to the short extracellular segment of gp41 which contains the Env membrane spanning domain and the cytoplasmic tail (Pierson et al., 2004). The short extracellular segment of gp41 contains the fusogenic peptide of HIV-1.

After cleavage, gp120 and gp41 assemble into a trimeric complex allowing for subsequent viral attachment, membrane fusion, and entry (Pierson et al., 2004).

Regulatory proteins: Tat and Rev. The two crucial regulatory proteins encoded by HIV-1 are trans-activator of transcription (Tat) and regulator of virion gene expression (Rev), shown in yellow in Figure 1.3. These proteins play key roles in viral transcription and replication. Tat and Rev are expressed early in infection. They are derived from multiply spliced RNAs generated from the same 5' splice donor. Tat is a 14 kDa protein that is a potent transcriptional activator for the HIV-1 promoter in the 5' LTR. Tat functions as a transcriptional elongation factor to greatly enhance processivity. Importantly, the HIV-1 LTR codes a transactivation response element (TAR) that is an RNA stem loop. Tat interaction with TAR allows for recruitment of host transcription factors and stabilization of the elongation complex. The TAR RNA stem loop is just downstream of the initial capped base of the HIV-1 9.6 kb transcript. (Seelamgari et al., 2004; Selby et al., 1989).

Rev is an 18 kDa protein required for nuclear export of unspliced viral RNAs. Rev mediated mRNA transport requires Rev binding to the Rev response element (RRE). Rev is required for expression of HIV-1 late genes, which includes the viral structural genes. The RRE is encoded in the gp41 env open

reading frame (ORF) and forms a highly stable RNA stem loop structure. Rev binding to RRE facilitates mRNA export and subsequent cytoplasmic expression of unspliced and once spliced transcripts. During the early phase of infection, low levels of Rev are produced such that only short multiply spliced transcripts are exported. These mRNAs encode Tat, Rev and Nef. Late in infection, there are higher levels of Rev expression such that Rev can suppress splicing and facilitate export of longer transcripts. Production of Rev suppressed its own production but the suppression is not complete as Tat and Nef multiply spliced messages continue to be produced (Malim et al., 1989; Selby et al., 1989).

Accessory proteins: Vif, Vpr, Vpu, and Nef. Viral infectivity factor (Vif) is a 23 kDa protein that interacts with host cellular factors that inhibit infection. Most importantly, Vif interacts with the cellular factors apolipoprotein B mRNA editing enzyme 3G (APOBEC3G). APOBEC3G is a cytidine deaminase that hypermutates viral DNA, which is known to be a host mechanism to inhibit HIV-1 replication. Vif counteracts this cellular inhibitory activity by binding APOBEC3G, inducing its proteasomal degradation, and preventing encapsidation (Marin et al., 2003; Sheehy et al., 2002; Sheehy et al., 2003). The most current model of Vif function is that Vif binds APOBEC3G, ElonginC, and Cullin5 which

in concert with Rbx2 and ElonginB forms an E3 ubiquitination complex (Stanley et al., 2008).

Viral protein R (Vpr) plays two main roles in HIV-1 pathogenesis. First, Vpr functions to transport the viral pre-integration complex (PIC) to the cell nucleus of the infected cell and then later induces host G2/M cell cycle arrest (Malim and Emerman, 2008). The PIC is the large complex containing the viral RNA/DNA, the viral structural protein and reverse transcriptase. It is the site of reverse transcription of the viral RNA genome to produce the double stranded DNA form, or provirus, that integrates into the host genome (Sherman and Greene, 2002). Vpr facilitates the nuclear import of the PIC early in infection (Bukrinsky and Haffar, 1999). G2/M cell cycle arrest occurs upon dephosphorylation of cdc25 resulting in the inactivation of the cdk1/cyclin B complex. Vpr functions to recruit PP2A to dephosphorylate cdc25, thus inducing cell cycle arrest in proliferating cells and subsequent cellular apoptosis (Hrimech et al., 2000).

The two best characterized functions of viral protein U (Vpu) in the viral life cycle are the degradation of CD4 and the enhancement of virion release. Vpu is translated from a bis-cistronic messenger RNA that also encodes Env. Based on the “leaky ribosome-scanning” model, it is thought that approximately half of

scanning ribosomes bypass the weaker Vpu initiation codon to initiate translation at the stronger Env initiation codon (Anderson et al., 2007).

In the absence of Vpu, newly synthesized CD4 forms an intracellular complex with gp160 that is sequestered in the ER. Retention of the CD4/Env complex leads to proteolytic digestion of proteins in the ER. Constitutive phosphorylation of Vpu by casein kinase 2 results in binding of Vpu to CD4-gp160 complexes, facilitating the release of bound gp120 into virions (Willey et al., 1992). Vpu acts by inducing polyubiquitination of CD4 leading to degradation via proteosomes by targeting CD4 to E3 ubiquitination complexes (Margottin et al., 1998).

Nef is a multifunctional protein that plays key roles in HIV-1 pathogenesis. Nef was originally named for negative factor based on the mistaken assumption that HIV-1 had a latent stage. Nef is a ~27-32 kDa myristoylated protein. Nef itself is not a kinase and has no known enzymatic activity. Nef is highly polymorphic in length and in sequence composition. The four most well characterized cellular activities of Nef are the downregulation of cell surface CD4, the downregulation of major histocompatibility complex I (MHC-I), the enhancement of viral infectivity, and the activation of p21-activated kinase 2 (PAK-2). Importantly, mutational analysis has demonstrated that these four

activities are genetically separable functions of Nef (Foster et al., 2001; O'Neill et al., 2006b).

The work described here has begun to define the effector domains for these four defined Nef functions. These domains consist of multiple effector domains including N-terminal myristoylation, SH3 binding, and a dileucine trafficking signal. Multiple effector domains have also been found that are unique to Nef. An additional complexity of Nef function is its ability to form dimers and higher oligomers (Arold et al., 2000).

Nef is thought to be a determinant of HIV-1 pathogenesis and disease progression. Moreover, the polymorphic nature and multifunctional activities of this protein make Nef a particularly challenging protein to study. The following chapters will review the most salient literature characterizing this nefarious protein.

Chapter 2: Literature Review of Nef *in vitro* Activities

CD4 downregulation by Nef. HIV-1 Nef downregulation of cell surface CD4 was the first in vitro activity of Nef characterized. Even before this protein was called Nef, it was reported that the 206 amino acid product of the F/3' ORF gene was capable of downregulating CD4 in CEM-T4 cells (Guy et al., 1987). Although this finding was subsequently challenged by Cheng-Mayer et al. CD4 downregulation by Nef has been unequivocally demonstrated in T cells (HPBALL), B cells (AA2), and monocytes (U937) transduced with HIV-1 SF2 Nef (Garcia and Miller, 1991). It is thought that this downregulation of Nef decreases the half-life of CD4 at the cell surface. Pulse-chase experiments have shown that Nef expressed in U937 cells increases CD4 degradation three-fold (Anderson et al., 1994).

The apparent role of CD4 downregulation in HIV-1 pathogenesis is to allow for a viral replicative advantage. Downregulation of CD4 by the virus is thought to be advantageous to prevent viral super infection (Benson et al., 1993). Moreover, a high level of CD4 on the cell surface is deleterious for the virus because the infectivity of released virions is reduced. The presence of CD4 can result in incorporation into the virion as a result of CD4/Env interaction. In this way the ability of a newly synthesized virion to infect would be compromised.

Lama et al. proposed an alternative model where Nef downregulation of CD4 allows for the production of more efficient virions. In the absence of Nef, there are exceedingly high levels of CD4 on the cell surface and HIV-1 Env accumulates at the plasma membrane such that Env is inefficiently incorporated into mature virions. The result is the release of virions with reduced infectivity (Lama et al., 1999). It is interesting to note that the existence of *nef* and *vpr* in the HIV-1 genome is necessary because of the use of CD4 as the primary receptor for cell entry. Overproduction of Env effectively retains its receptor in the ER allowing for virions of high infectivity to be produced at the cell surface.

Nef downregulation of CD4 has been proposed to be mediated through Nef directly binding to the cytoplasmic tail of CD4. The cytoplasmic tail of CD4 is necessary and sufficient for Nef mediated downregulation of CD4 (Anderson et al., 1994; Garcia et al., 1993). While CD4 downregulation by phorbol esters requires serine phosphorylation of the CD4 tail, Nef downregulation of CD4 is independent of serine phosphorylation (Garcia and Miller, 1991). This interaction has been further dissected by mutational analysis and NMR studies. Grzesiek et al. demonstrated Nef directly binds the QIKRLL peptide of the CD4 cytoplasmic tail (Grzesiek et al., 1996).

Mutational analyses have been performed to map the functional domains on Nef and on CD4 that are required for Nef mediated CD4 downregulation. Early

studies first identified two discontinuous regions on Nef critical for CD4 downregulation, specifically residues 96-144 and residues 175-186 (Hua and Cullen, 1997). The cytoplasmic domain of CD4 has also been dissected to determine the sites on CD4 dictating Nef mediated downregulation. Importantly, different amino acids on CD4 are required for Nef mediated downregulation by different Nef proteins from HIV-1, HIV-2 and SIV. HIV-1_{SF2} or HIV-1_{NL4-3} downregulate CD4 efficiently if positions 405, 406, or 407 on the cytoplasmic domain of human CD4 are mutated. However HIV-2 and SIV do not tolerate such mutations. HIV-2 and SIV Nef require a glutamic acid at position 405 and a methionine at position 407 of human CD4 (Hua and Cullen, 1997). As a result of these specific amino acids on CD4, SIV Nef cannot downregulate murine CD4 (Foster et al., 1994).

Nef downregulation of CD4 is dictated by additional key regulatory domains on Nef. Nef contains a canonical dileucine motif, $_{160}$EXXXLL$_{165}$, on the C-terminal flexible loop, which suggests a role for adaptor proteins, AP-1, AP-2, and AP-3. These two leucines are necessary for CD4 downregulation and association with the clathrin-associated adaptor protein 2 (AP-2) (Bresnahan et al., 1998; Greenberg et al., 1997). In addition to $_{160}$EXXXLL$_{165}$, the $_{174}$(E/D)D$_{175}$ diacidic motif has also been shown to be necessary for AP-2 interaction and CD4 downregulation (Lindwasser et al., 2008; Piguet et al., 1999). This interaction was

demonstrated in vitro by yeast three hybrid, GST pulldown, and surface plasmon resonance experiments. The E in $_{160}$EXXXLL$_{165}$ is not significantly involved (Craig et al., 1998). Instead, AP-2 binding to Nef requires the conserved $_{174}$(E/D)D$_{175}$ motif which is specific for AP-2. Based on these data, the Bonifacino group proposed that the Nef/AP-2 complex regulates CD4 downregulation by enhancing endocytosis of CD4 (Lindwasser et al., 2008). Although Nef binding to AP-2 involves adjacent leucines the binding is non-canonical given the requirement for two acidic residues to the C-terminal side of the two leucines. Regions on Nef other than residues 160-175 have also been identified to be key mediators of CD4 downregulation. Residues $_{57}$WL$_{58}$ of the HIV-1 Nef domain are necessary for Nef binding to CD4 (Grzesiek et al., 1996). Aspartate 123 of HIV-1 Nef has also been shown to be required for CD4 downregulation, such that mutation of position 123 to glutamate complete ablates Nef mediated CD4 downregulation (O'Neill et al., 2006b).

Nef interaction with p56^{Lck} has also been proposed to play a role in CD4 downregulation. p56^{Lck} is a Src family tyrosine kinase that plays synonymous roles in T cell signaling. Nef has been demonstrated to interact with p56^{Lck} (Baur et al., 1997; Greenway et al., 1996). This interaction blocks CD4 endocytosis (Rhee and Marsh, 1994). The 38 amino acid cytoplasmic tail of CD4 associates with the N-terminus of p56^{Lck}, this interaction between the cytoplasmic tail of

CD4 and $p56^{Lck}$ is stronger than the interaction of Nef with the cytoplasmic tail of CD4 (Geleziunas et al., 1994). Unfortunately, the role of $p56^{Lck}$ in Nef mediated CD4 downregulation remains unresolved. In particular, it is not known how Nef disengages $p56^{Lck}$ from CD4. In the presence of Nef, the intracellular distribution of $p56^{Lck}$ is altered, however this was independent of Nef induced CD4 downregulation (Thoulouze et al., 2006).

The mechanism of CD4 downregulation by Nef involves CD4 degradation via the endosomal-lysosomal pathway. Treatment of Nef expressing cells with lysosomal inhibitors resulted in CD4 accumulation in lysosomes indicating that mechanism of Nef downregulation of CD4 includes CD4 degradation in lysosomes (Luo et al., 1996). Moreover, Nef increases the number of CD4 containing clathrin-coated pits (Foti et al., 1997). In this model, Nef association with the cytoplasmic tail of CD4 and AP-2 forms a CD4-specific clathrin coated vesicle that is targeted to early endosomes. This protein complex is then transported to late endosomes via β-COP and finally degraded in lysosomes (Piguet et al., 1999).

MHC-I downregulation by Nef. Nef downregulation of MHC-I is a key mechanism for HIV-1 evasion of host immune function. The loss of cell surface expression of MHC-I prevents the presentation of viral antigens to cytotoxic T

lymphocytes (CTLs), thus HIV-1 infected cells avoid recognition and CTL killing. HIV-1 Nef was first demonstrated to be responsible for downregulation of cell surface MHC-I in HIV-1 infected T cells by Schwartz et al. (Schwartz et al., 1996).

Similar to CD4 downregulation, the mechanism of Nef-mediated MHC-I downregulation involves the disruption of host cellular trafficking pathways. One model proposes that Nef downregulation of MHC-I requires Nef binding to the cytosolic adaptor protein phosphofurin acidic cluster sorting (PACS-1). In this model proposed by Thomas et al., PACS-1 is thought to bind HIV-1 Nef via an $_{62}$EEEE$_{65}$ acidic cluster (Blagoveshchenskaya et al., 2002; Hung et al., 2007). The PACS-1/Nef complex is localized to the TGN where Nef by means of a PxxP ($_{72}$PQVP$_{75}$) recruits and activates a src family kinase (SFK) resulting in downstream activation of phosphoinositol-3 kinase (PI3K). PI3K increases plasma membrane levels of phosphoinositol-3,4,5-triphosphate (PIP_3), such that elevated PIP_3 recruits ARNO to GTP load Arf6. GTP loaded Arf6 then increases the endocytosis of MHC-I via a non-clathrin mediated pathway thereby bringing MHC-I to the TGN. More recently however, this model has been challenged by demonstrating that HIV-1 Nef binding to PACS-1 is too weak to be physiologically relevant (Baugh et al., 2008).

In contrast, an alternative model from the Collins group proposes that Nef acts early in the secretory pathway by binding the hypophosphorylated MHC-I cytoplasmic tail. In the secretory pathway, nascent MHC-I assembled at the endoplasmic reticulum (ER) with the β2-microglobulin and the antigen peptide. This complex is then transported to the trans-Golgi network (TGN) where MHC-I cytoplasmic tail is phosphorylated. Nef binding to hypophosphorylated MHC-I sequesters MHC-I at the TGN, which allows for binding to AP-1. This complex is then targeted for degradation through the endosomal-lysosomal pathway (Kasper et al., 2005; Roeth and Collins, 2006).

Details of the involvement of AP-1 in MHC-I downregulation by Nef has been further characterized. Two independent groups have demonstrated that the cytoplasmic domain of MHC-I alone does not bind AP-1. The Guatelli group showed that the MHC-I cytoplasmic domain alone has no intrinsic ability to bind AP-1, however Nef fused to the MHC-I cytoplasmic domain (MHC-I CD-Nef) was able to bind AP-1. The binding of MHC-I CD-Nef to AP-1 required Y320 of the MHC-I cytoplasmic tail and $_{62}$EEEE$_{65}$ of Nef. Importantly, the μ subunit of AP-1 is required for binding to MHC-I CD-Nef (Noviello et al., 2008). These findings corroborate data from the Collins group, who also demonstrated the μ1 subunit of AP-1 is required for binding to the cytoplasmic tail of MHC-I. Similar to the previous studies, these authors also characterized the importance of Y320 in

AP-1 binding to Nef. In their model, Y320 is proposed to be the key residue on the MHC-I cytoplasmic tail that forms the Nef binding pocket and sequentially allows for MHC-I binding to AP-1, as depicted in Figure 2.1. Furthermore, it is thought that M20 and the $_{62}$EEEE$_{65}$ acidic domain of Nef stabilize the interaction between MHC-I and AP-1 (Wonderlich et al., 2008).

In addition to the previously described $_{62}$EEEE$_{65}$ acidic cluster, P78 of the highly conserved Nef polyproline helix was determined to be a key for the formation of the Nef/MHC-I cytoplasmic tail/AP-1 complex (Noviello et al., 2008; Yamada et al., 2003). It has previously been suggested that Nef downregulation of MHC-I requires an intact SH3 binding motif on Nef based on mutating the prolines of PxxP to alanines (Greenberg et al., 1998). Nef contains a canonical class II SH3 binding domain as defined by Mayer, this segment consists of the highly conserved $_{72}$PQVPLR$_{77}$ (Mayer, 2001). However, it was later shown that Nef P78 regulates MHC-I downregulation in an SH3 independent mechanism (Casartelli et al., 2006). It is known that mutations in the Nef polyproline motif will have deleterious effects on multiple functions of Nef, but in the case of MHC-I downregulation by Nef, another group has identified P78 as the critical residue while P72 and P75 are not as significant (Yamada et al., 2003).

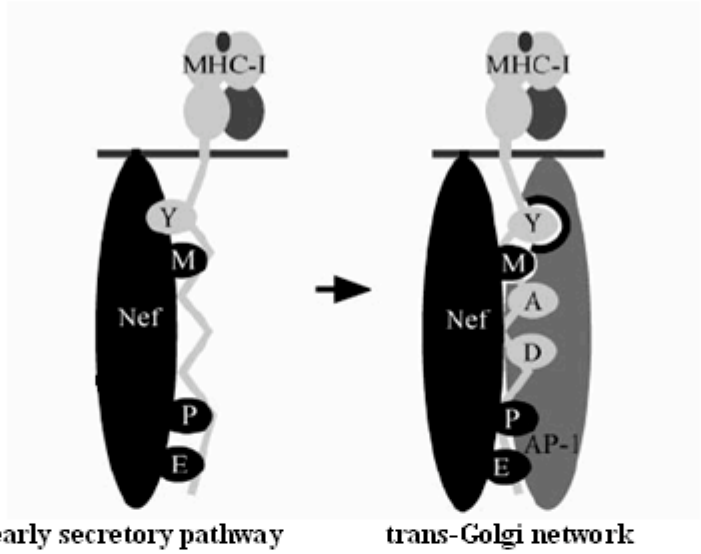

Figure 2.1 Proposed model for Nef/MHC-I/AP-I ternary complex. The model for Nef binding to MHC-I and then recruitment of AP-1 as proposed by the Collins group. In their model, Nef (black) binding to MHC-I (light grey) is mediated by the Nef polyproline helix (designated P), the acidic domain (designated E) and methionine 20 (designated M). These regions on Nef interact with the cytoplasmic tail of MHC-I via tyrosine 320 (designated Y). The binding of Nef to the cytoplasmic tail of MHC-I occurs early in the secretory pathway, then subsequent binding to AP-I (dark grey) occurs in the trans-Golgi network. Figure modified from Wonderlich et al.

Nef enhancement of viral infectivity. The ability of HIV-1 Nef to enhance virus infection in single round infectivity assays is another important in vitro activity of Nef. The primary observation was that a *nef*-defective virus exhibited lower infectivity, however this decrease in infectivity could be complemented by Nef expression in trans (Chowers et al., 1994; Miller et al., 1994). It is thought that Nef enhancement of infectivity may allow for more efficiently infectious virions, however the exact cellular mechanism(s) by which this occurs remains poorly understood. The effect is observed when HIV-1 is produced by cells lacking CD4. Therefore, this function of Nef is independent of CD4 downregulation.

Nef enhancement of HIV-1 infectivity is thought to occur early in the viral life cycle, more specifically acting after viral entry but before viral protein synthesis. Early reports indicated that Nef may stimulate proviral DNA synthesis by increasing the efficiency of reverse transcriptase in the infected cell, thereby reducing the percentage of abortive infections (Aiken and Trono, 1995; Schwartz et al., 1995). It has been proposed that incorporation of Nef protein into the virus particle and delivery to the target cell may mediate the increased infectivity (Pandori et al., 1996; Welker et al., 1998; Welker et al., 1996).

More recently, other host cellular targets such as the actin cytoskeleton and Dynamin-2 have been proposed to regulate Nef enhancement of infectivity. In

one model, Campbell et al., propose that upon plasma membrane fusion the virus must penetrate a dense layer of cortical actin. HIV-1 Nef is thought to mediate actin trafficking and reorganization to facilitate viral membrane fusion (Campbell et al., 2004). Alternatively, Pizzato et al. proposed that the specific interaction between HIV-1 Nef and Dynamin 2 (Dyn2) is required for the infectivity enhancement activity of Nef (Pizzato et al., 2007). Dyn2 is a GTPase involved in clathrin-mediated endocytosis, thus it was demonstrated that this Dyn2/Nef-mediated enhancement of infectivity requires clathrin for endocytosis.

Nef enhancement of infectivity is also been proposed to occur through a proteasome dependent mechanisms. HIV-1 infectivity is enhanced by proteasome inhibition (Schwartz et al., 1998; Wei et al., 2005b). These studies were extended to further demonstrate that HIV-1 infectivity is increased by Nef across different HIV-1 isolates and in the presence of lysosome and proteasome inhibitors. Strikingly, HIV-1_{SF2} infectivity is increased 400-fold upon treatment with both the lysosomal inhibitor, bafilomycin A1, and the irreversible proteasome inhibitor, lactacystin (Wei et al., 2005b). Treatment of cells with proteasome inhibitors such as MG132 or lactacystin was shown to enhance cellular susceptibility to infection by *nef*-defective HIV-1 (Qi and Aiken, 2007). In this model, it is proposed that Nef enhancement of HIV-1 viral infectivity occurs by "modifying the virion during particle assembly" such that the virus is less susceptible to proteasomal

degradation. These authors later demonstrate Nef enhancement of infectivity is mediated by association with viral assembly. Using a cyclophilin A-Nef fusion protein, the authors show that this cyclophilin A-Nef fusion is incorporated into virions and co-purifies with HIV-1 cores (Qi and Aiken, 2008).

Two effector domains are important for enhancement of infectivity. These are the $_{72}$PQVPLR$_{77}$ and $_{160}$EXXXLL$_{165}$. Because of the lack of a fine structure mutational analysis, it is not clear that the $_{72}$PQVPLR$_{77}$ domain is acting as an SH3 binding site (Goldsmith et al., 1995). On the other hand, the $_{160}$EXXXLL$_{165}$ motif does appear to be canonical and involves Nef binding to AP-1 and/or AP-3 (Coleman et al., 2006).

Summary and Perspectives. Altogether, the studies highlighted in this literature review demonstrate the complex nature of Nef cellular functions. Nef has many roles in cellular signaling as well as regulating key immune functions. The ultimate goal of Nef research is to fully characterize the role of Nef in HIV-1 pathogenesis. The ultimate goal is to determine whether Nef will be a useful target in the development of anti-viral therapies. .

Chapter 3: Literature Review of Nef Phosphorylation

Initial reports of Nef phosphorylation. Four years after the discovery of HIV-1, *in vitro* phosphorylation of the HIV F/3' orf encoded protein was described. This F protein was known to be N-terminally myristoylated and the F-protein was expressed in BHK-21 cells infected with a recombinant vaccinia virus vector. This F-protein was basally phosphorylated and this phosphorylation was increased in the presence of the protein kinase C (PKC) activator TPA (12- O-tetradecanoylphorbol-13-acetate). These early data first characterized a potential PKC mediated Nef phosphorylation. Based on partial sequence homology to pp60-src protein and epidermal growth factor (EGF) receptor, it was hypothesized that threonine 16 of F protein from HIV-1_{Bru} and HIV-2_{Rod} was a PKC site (Guy et al., 1987). However the biological significance of this putative threonine 16 site was subsequently discounted.

Bacterially expressed Nef was next shown to be phosphorylated by a cellular kinase. Phosphorylated Nef bands were observed upon incubation of GST-BH10 Nef immobilized on glutathione agarose beads with cell lysates from Jurkat, HeLa, and U937 cells and $^{\gamma 32}$P-ATP. Phosphoamino acid TLC analysis demonstrated this GST-Nef phosphorylation occurred predominantly on serine residues. This phosphorylation was postulated to be mediated by PKC based on

treatment with the PKC inhibitor, bisindolylmaleimide. In the absence of cell extracts, GST-Nef phosphorylation was observed using commercially available purified PKC (Coates and Harris, 1995).

Coates et al. further extended these studies to demonstrate PKC mediated Nef phosphorylation in HeLa and Jurkat cells. This metabolic phosphorylation of Nef was increased upon stimulation with phorbol esters. Phosphoamino analysis confirmed previous in vitro data showing the phosphorylation occurred primarily on serine residues. Treatment of cells with bisindolylmaleimide inhibited phosphorylation, conversely Nef phosphorylation was unaffected upon treatment with MAPK (PD98059) or cAMP dependent kinase (PKIα) inhibitors. Finally, the authors demonstrated that myristoylated Nef had increased phosphorylation compared to the non-myristoylated G2S mutant (Coates et al., 1997). It is important to note that subtle differences in the experimental systems used throughout these different studies accounts for some of the conflicting data in this field.

HIV-1 Nef phosphorylation in human T cells. Similar to the previously described studies, Luo et al. also demonstrated Nef phosphorylation but in a more physiologically relevant setting. Basal Nef phosphorylation was observed in HuT-78 T cell line acutely infected with HIV-1_{SF2}. This basal phosphorylation was

increased upon treatment phorbol myristate acetate (PMA) and phytohemagglutinin (PHA) to activate the T cells. Basal and PMA induced phosphorylation was also demonstrated in HuT-78 cells transduced by an amphotropic retroviral vector to express SF2 Nef. Consistent with previous findings from other groups, phosphoamino analysis showed that Nef was phosphorylated predominantly on serine residues. Treatment of Nef transduced HuT-78 cells with bisindolylmaleimide abrogated PMA induced phosphorylation while phosphorylation was unaffected by the PKA inhibitor, H89.

The most important findings of Luo et al. that set it apart from previously published studies was the establishment of the functional consequence of PMA induced Nef phosphorylation. CD4 downregulation was assayed using HeLa.H4 cells which express the CD4 mutant (SXXA, SXXA, SXXA). These serine mutations in the cytoplasmic tail of CD4 render CD4 insensitive to PMA downregulation (Garcia and Miller, 1991; Shin et al., 1990). Thus, in HeLa.H4 cells, it was argued that any observed CD4 downregulation is mediated only by Nef. In Nef expressing HeLa.H4 cells, CD4 downregulation more than doubled upon treatment with PMA suggesting that PMA-induced Nef phosphorylation potentiates CD4 downregulation by Nef (Luo et al., 1997a). Altogether, this study characterized Nef phosphorylation and suggested a functional significance in CD4 downregulation.

PKC phosphorylation of CD8-Nef. Work from the Baur group has demonstrated PKC phosphorylation of a CD8-Nef hybrid consisting of a fusion protein between the extracellular and transmembrane domain of CD8 and the N-terminal 35 amino acids of SF2 Nef. CD8-Nef was phosphorylated by members of the novel PKC family, PKCδ and PKCθ rather than conventional or atypical PKCs, such as PKCα, PKCβ, PKCγ, or PKCζ. The authors report that this phosphorylation occurs on S6, which is part of SF2 Nef's membrane myristoylation signal. Using an S6A mutant, the authors suggest that CD8-Nef phosphorylation by PKCδ or PKCθ has downstream effects on viral infectivity and subcellular localization (Wolf et al., 2008).

SIV Nef phosphorylation. In addition to the aforementioned HIV-1 Nef phosphorylation, SIV Nef phosphorylation has also been described. SIV Nef phosphorylation was first demonstrated in CEMx174 cells expressing the prototypical SIVmac239 Nef and a macrophage tropic neurovirulent clone, SIV/17E-Fr (Barber et al., 1998). This study was later extended to identify casein kinase 2 (CK2) as the cellular kinase responsible for SIV Nef phosphorylation. Recombinant CK2 was able to phosphorylate recombinant GST-SIV/17E-Fr Nef. Using S12P and D15A mutants, the authors suggest that S12 and D15 are

necessary for SIV/17E-Fr Nef phosphorylation. Treatment of cells with 4,5,6,7-tetrabromobenzotriazole (TBB), a specific CK2 inhibitor reduced SIV/17E-Fr Nef phosphorylation (Caples et al., 2006). It is not clear that these results relate to HIV-1 Nef phosphorylation. SIV Nefs and HIV-1 Nefs share a number of functions but do so by mechanistically distinct pathways (Kirchhoff et al., 2008).

Summary and Perspectives. These studies highlight the confounding Nef phosphorylation literature. It will be important to further dissect the regulatory determinants of Nef phosphorylation. Specifically, one objective of the work in this thesis was to further characterize Nef phosphorylation to first determine the specific serine residues on Nef phosphorylated and second, to further investigate the functional consequence of Nef phosphorylation.

Chapter 4: Literature Review of Nef Activation of PAK-2

First identification of a Nef associated kinase. Nef binding to a host cellular kinase was first described by Sawai et al. and subsequently also characterized by other groups (Luo et al., 1997b; Renkema et al., 1999; Sawai et al., 1994). A CD8-HIV-1 Nef chimera was expressed in Jurkat T cells and Nef co-immunoprecipitates incubated with $^{\gamma 32}$P-ATP revealed two phosphorylated bands with molecular weights of 62 kDa and 72 kDa. Phosphoamino acid analysis revealed that these two co-immunoprecipitated bands were phosphorylated on serine residues (Sawai et al., 1994). This kinase activity was further demonstrated to be associated with SIV Nef immunoprecipitates and that the C-terminus of Nef was important for binding to the yet to be determined cellular kinase (Luo et al., 1997b).

The Nef associated kinase is PAK-2. The Nef associated cellular kinase was suspected to be a member of the p21 activated kinase (PAK) family based on immunoprecipitation of the Nef associated kinase activity using an antibody against amino terminus of PAK-1 (Sawai et al., 1996). Based on immunoprecipitation with this amino terminus PAK-1 specific antibody, it was hypothesized at this point that this Nef associated kinase was either PAK-1, PAK-

2, or PAK-3. Fackler et al. subsequently identified the Nef associated kinase activity to be PAK-1 (Fackler et al., 2000) while Renkema et al. identified this activity to be PAK-2 (Renkema et al., 1999). PAK-1 (65 kDa) and PAK-2 (62 kDa) are highly homologous, and cross-reactivity of a PAK-1 interfering fragment accounted for the misidentification of PAK-1 as the Nef associated kinase (Rauch et al., 2008). PAK-2 was ultimately determined to be the target kinase based on in gel proteolytic digestion (Arora et al., 2000). PAK-2 but not PAK-1 is cleaved by caspase 3 in vitro and the Nef associated kinase activity is caspase 3 sensitive. This specificity was demonstrated in multiple different Nef alleles, specifically HIV-1 SF2, HIV-1 NL4-3, and SIVmac239 and in multiple different T cell (CEM, HuT-78, and Jurkat) and monocytic cell (U937 and THP-1) lines (Arora et al., 2000). Monospecific antiserum for PAK-1 and PAK-2 have recently become commercially available. Unpublished work from Wei et al. has demonstrated that immunoprecipitates with these antibodies have confirmed that Nef activates PAK-2 but not PAK-1.

Specificity and abundance of the Nef/PAK-2 complex. Nef association and activation of PAK-2 is highly specific. The strength and specificity of this interaction is apparent given the resistance of the Nef/PAK-2 interaction to 1M $MgCl_2$ washings. Moreover it was demonstrated that Nef activates PAK-2 in a

low abundance complex (Sawai et al., 1995). In order to investigate the stoichiometry of the Nef/PAK-2 complex, successive immunodepletions experiments were performed. It was shown that most of the activated PAK-2 was bound to Nef however there was also a lesser fraction of activated free PAK-2 not associated with Nef. Even when PAK-2 is overexpressed, the Nef/PAK-2 complex is found in low abundance. These data suggested that there must be other important and limiting cellular factors present in the Nef/PAK-2 complex (Arora et al., 2000).

Cellular components of the macromolecular Nef/PAK-2 complex.

Many host cellular proteins have been reported to be members of the Nef/PAK-2 complex. Co-expression of constitutively active Cdc42 and Rac1 increased Nef associated PAK-2 activity while the dominant negative forms of Cdc42 and Rac1 inhibited kinase activation (Lu et al., 1996). Moreover, it was demonstrated that Nef activation of PAK-2 required activated endogenous Cdc42 and Rac1 in a cell-free system (Raney et al., 2005). It was also hypothesized that Nck could be one of the limiting cellular factors involved in Nef activation of PAK-2 given the roles Nck plays as a SH3 adaptor protein. Mutation of the PxxP motif in PAK-2 to AxxA prevented Nck binding, however this did not affect Nef activation of PAK-2 thus Nck was ruled out as a necessary component of the Nef/PAK-2 complex

(Renkema et al., 2001). β-PIX was also hypothesized to be a part of the Nef/PAK-2 complex by co-immunoprecipitation experiments with the β-PIX-associated protein p95PK (Brown et al., 1999). However this was later refuted based on experiments demonstrating that mutations in PIX-binding motif in PAK-2 had no effect on the ability of PAK-2 to associate with Nef, thus excluding β-PIX and its associated factors as essential components of the Nef/PAK-2 complex (Renkema et al., 2001).

Work by Fackler et al. has proposed the involvement of Vav in Nef activation of PAK-2. Vav was first identified to be a component of what is now known to be the Nef/PAK-2 complex by immunoprecipitation of Vav with a CD8-Nef hybrid. This interaction was further mapped to the PxxP motif of Nef and the C-terminal SH3 domain of Vav. Expression of a dominant negative Vav inhibited Nef activation of PAK-2 (Fackler et al., 1999). From these data, it was hypothesized that Vav mediates cellular cytoskeletal rearrangements upon Nef activation of PAK-2. This model was further extended to demonstrate that Nef recruits Vav1 to detergent-insoluble membrane microdomains. Biochemical fractionation and confocal microscopy showed the presence of Vav1 in these membrane microdomains, but not β-PIX (Rauch et al., 2008).

Cellular localization of the Nef/PAK-2 complex. The cellular

localization of Nef activated PAK-2 has also been extensively investigated. Nef activation of PAK-2 is thought to occur at cellular membranes as a result of the requirement for myristoylation. Nef activation of endogenous PAK-2 in a rabbit reticulocyte cell free system required the presence of canine microsomal membranes. Moreover, the Nef G2A myristoylation mutant was unable to activate PAK-2 in this system (Raney et al., 2005). Nef activation of PAK-2 has also shown to occur in Triton X-100 detergent insoluble microdomains. Based on Optiprep lipid raft flotation analysis, PAK-2 activated by Nef co-fractionated with the lipid raft marker GM1 (Krautkramer et al., 2004). OTG-mediated raft disruption interfered with activation of PAK-2 by Nef. GFP labeled Nef clustered in lipid rafts using a CTx antibody as a GM1 marker. In this model, Krautkramer et al propose that Nef activates PAK-2 through recruitment to lipid rafts and subsequently activates the T cell receptor signaling cascade (Krautkramer et al., 2004).

Downstream signaling of PAK-2 activated by Nef. The physiological downstream consequence of Nef activation of PAK-2 remains controversial. Nef activation of PAK-2 has been proposed to mediate cellular apoptosis as well as T cell signaling (Fenard et al., 2005; Geleziunas et al., 2001; Krautkramer et al., 2004; Wolf et al., 2001). Merlin (Moesin, Ezrin, and Radixin-Like protein) has

been reported to be a direct downstream substrate of PAK-2 activated by Nef. Merlin is encoded by the Nf2 tumor suppressor gene and is a negative regulator of Rac1 signaling. Nef activated PAK-2 phosphorylates merlin on serine 518 which would relieve the growth suppressive state of merlin allowing for cellular proliferation (Wei et al., 2005a). These data support a model where Nef activation of PAK-2 results in downstream signaling to allow for cytoskeletal rearrangements necessary for T cell activation.

Characterization of the effector domain on Nef responsible for PAK-2 activation. Several mutations have been found to abrogate Nef activation of PAK-2. The G2A mutation which prevents myristoylation greatly reduces PAK-2 activation but this is observed for nearly all Nef in vitro functions. Mutation of the adjacent arginines to adjacent leucines at amino acid positions 105 and 106 (RR/LL) also prevents PAK-2 activation (O'Neill et al., 2006b). When other Nef functions were tested it was not surprising that partial defects were observed in CD4 downregulation, MHC-I downregulation and enhancement of infectivity. The drastic nature of these mutations makes the observations difficult to interpret mechanistically (O'Neill et al., 2006b).

Subsequent in vivo studies with rhesus macques infected with SIV_{mac239} questioned the findings of Sawai et al (Kestler et al., 1991; Sawai et al., 1996). On

the basis of these observations, the RR/LL mutation was used for in vivo infection studies to argue for the requirement of PAK-2 activation for the pathogenicity of the SIV_{smm} molecular clone SIV_{mac239} in rhesus macques (Sawai et al., 1996). Moreover, the RR to LL mutation also reduced the half-life of the Nef protein (Luo et al., 1997b). Altogether, the role of amino acid positions 105 and 106 on PAK-2 activation remains unclear.

The PxxP motif of Nef has also been suggested to be of decisive importance for Nef induced PAK-2 activation (Greenberg et al., 1998; Manninen et al., 1998). Many groups have used the AxxA mutation to investigate the role of the polyproline helix in Nef function. However, as with the previously mentioned RR/LL mutation, the AxxA mutation is too drastic such that the results from this mutation have not yielded mechanistically interpretable results. Moreover, the misuse of this AxxA mutation for mechanistic interpretation is particularly evident in studies of MHC-I downregulation by Nef (Greenberg et al., 1998).

Mutational dissection of the specific amino acids involved in Nef activation of PAK-2 have mapped a unique interaction domain on Nef responsible for PAK-2 activation. The HIV-1 primary isolate 233 is defective for PAK-2 activation (Foster et al., 2001; Luo et al., 1997b). This isolate exhibited a phenylalanine to isoleucine substitution at amino acid position 191. Based on this polymorphism, detailed mutational analysis was initiated to identify other amino acids interacting

with phenylalanine 191. An extensive collection of 1,643 HIV-1 subtype B Nef sequences was compiled to perform the subsequent structure-function analysis. The F191I mutation is specific for defective PAK-2 activation, and has no effect on CD4 or MHC-I downregulation or enhancement of infectivity (Foster et al., 2001). Thus mutations were introduced at amino acid 191 to leucine, arginine, tyrosine, and isoleucine. Mutation of phenylalanine 191 to any of these residues completely ablated PAK-2 activation. In a parallel experiment, various HIV-1 subtype E Nef primary isolates were analyzed for their ability to activate PAK-2. All five of the HIV-1 subtype E Nef primary isolates examined were able to activate PAK-2, and all of these Nefs have an arginine at amino acid 191. Based on these data and sequence comparisons between subtype B and subtype E, compensatory sites on Nef for the arginine 191 mutation were predicted (O'Neill et al., 2006b).

A particularly strong association was noted between position 89 and 191. In a detailed analysis, residues 89 and 191 were determined to be components of an interaction domain responsible for PAK-2 activation with lesser contributions from position 85 and 188. Histidine 89 and phenylalanine 191 are highly conserved in subtype B Nefs, however in subtype E Nef phenylalanine 89 and arginine 191 predominate. Given subtype E Nefs also activate PAK-2, it appears at least two different interaction domains are present in HIV-1 Nefs. Substitution

of all four residues in a subtype B SF2 Nef with residues that predominant in subtype E Nefs (F85, F89, A188 and R191) to generate a fully functional subtype E PAK-2 interaction surface in a subtype B background (O'Neill et al., 2006b). Altogether, these data highlight the structural plasticity of Nef in the activation of PAK-2.

Summary and Perspectives. Based on these studies, it is clear that the activation of PAK-2 by Nef is complex. Work by O'Neill et al. has provided important insights into the structural determinant of PAK-2 activation. However, it remains unclear what other cellular factors are involved, specifically what other cellular factors interact with the Nef/PAK-2 complex. Work in this thesis will further dissect this PAK-2 effector domain on Nef.

Chapter 5: Essential Methodology

Cell culture. Human CEM and HuT-78 cells were cultured in 5% CO_2 in RPMI 1640 (Sigma R8758) medium supplemented with 10% fetal bovine serum (SAFC Biosciences 12303C, heat inactivated for HuT-78), 50 IU penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Cellgro 30-009-CI) and 1 mM sodium pyruvate (Cellgro 25000CI). HEK 293T cells were cultured in 10% CO2 in Dulbecco's Modified Eagle Medium (Sigma D5796) supplemented with 10% fetal bovine serum (SAFC Biosciences 12303C), 50 IU penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Cellgro 30-009-CI). 293T cells were split with trypsin EDTA (Cellgro 25-053-Cl).

DNA plasmid preparation. Briefly, 500 mL of Luria-Bertani broth were used to grow overnight cultures of DH5α competent bacteria transformed with the given DNA of interest. Plasmid DNA was isolated using Qiagen Tip 500 columns (Qiagen 10063). DNA was resuspended and stored in Tris-EDTA. DNA was quantitated by absorbance at 260 nm on a Beckman640B Spectrophotometer.

Transfections. HEK 293T were transfected using Lipofectamine 2000 (Invitrogen 11668-019). Briefly, 800,000 293T cells per well were plated without

antibiotics in a six-well plate one day prior to transfection such that the cells were at 90% confluency upon transfection. 4 μg DNA was resuspended in 250 μL Opti-MEM (Gibco 31985-070) in one tube and 10 μL Lipofectamine 2000 was resuspended in 250 μL Opti-MEM in a separate tube. After five minutes, the DNA suspension and the Lipofectamine 2000 suspension were gently mixed and incubated at room temperature for 20 minutes. Next, the mixture was added to the cells. 16 hours later, the media was replaced with fresh media containing antibiotics. 24 hours later cells were harvested for subsequent experimentation.

Transduction. Cells were transduced to express only the neomycin phosphotransferase gene (neo^r) or Nef and neo^r using a murine retroviral transduction system (Garcia and Miller, 1994). Nef isolates were cloned into the retrovirus vector pLXSN as EcoRI fragments as previously described (Garcia and Miller, 1991). pLXSN and pEQPAM were co-transfected into 293T cells by the calcium phosphate method to generate amphotropic vectors. At 36 to 48 h after transfection, vector-containing media were collected for transduction of cells. 24 well plates were coated with 40 μg Retronectin (Takara Bio Inc.), followed by 500 μl 2% BSA in phosphate buffered saline (PBS; Gibco Life Technologies; Rockville, MD) for 30 minutes at RT. Wells were then rinsed with serum free RPMI and incubated with 500 μl viral supernatants twice for 1 hour at 37° C.

Target cells (300,000) in complete RPMI were then added to each well. The next morning an additional 500 µl of viral supernatant was added to each well. 24 hours later, cells were pelleted and resuspended in complete RPMI with 1.5 mg/ml G418 for selection.

Preparation of whole cell lysates. Ten million transduced HuT-78 or CEM cells or an entire well of a six-well plate of transfected 293T cells (500 µL of trypsin were added to each well of 293T cells) were collected by centrifugation at 4°C and washed twice in 5 mL of ice cold PBS, centrifuged at 1500 rpm for 5 minutes at 4°C (CR422 Jouan Tabletop Centrifuge; 770 rotor) and lysed with complete lysis buffer (500 µL) at 4°C. Lysis buffer (50 mL) consisted of 50 mM Tris pH 8.0, 10% glycerol, 100 mM NaCl, 25 mM NaF, 2 mM Na_3VO_2, 20 mM β-glycerophosphate, 25 mM benzamidine, 2 mM EDTA pH 8.0, and 0.5% IGEPAL CA-630. Complete lysis buffer was supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and one mini protease inhibitor tablet (Roche 11836153001). Lysates were cleared in a microcentrifuge for 10 minutes at 13,000 rpm at 4°C to separate the detergent insoluble debris for the soluble protein. Protein concentration of the supernatant was determined by spectrophotometry using IgG protein standards in Bio-Rad Protein Assay reagent (BioRad #500-0006) diluted 1:5.

Western blotting. Whole cell lysate (200 μg) in 1X SDS loading buffer was boiled for five min at 95°C. (Stock solutions of 5X SDS were comprised of the following components: 5 mL 1M Tris, pH 6.8, 5 mL glycerol, 0.05 g bromophenol blue, and 1 g SDS. This mixture was heat at 95^{o}C for 5 minutes and diluted in 20% β-mercaptoethanol.) Proteins were then loaded on either a 10 or 7.5 % acrylamide gel for nine hours at 100 volts (900 volt hours) using the BioRad Power Pac300 (Model # 1655050) as a power source.

The resulting gel was soaked in transfer buffer for 10 min and transferred to a nitrocellulose membrane (Amersham RPN303E) on a BioRad Trans-Blot SD Cell. The 10% acrylamide gel was comprised of a resolving layer (12.3 mL dH_2O, 8.5 mL of 40% acrylamide/1% bisacrylamide, 12.4 mL 1 M Tris, pH 8.8) and a stacking layer (15.5 mL dH_2O, 2.5 mL 1M Tris, pH 6.8, 2 mL of 40 % acrylamide/1% bisacrylamide). Polymerization of the resolving layer was initiated with 165 μL (150 μL for the stacking layer) of 10% ammonium persulfate (APS) and 22 μL (20 μL for the stacking layer) of 1,2-bis(dimethylamino)ethane (TEMED). The 18% acrylamide gel was comprised of an 18% resolving layer (14.9 mL of 40 % acrylamide/2% bisacrylamide, 12.4 mL 1M Tris, pH 8.8, 5.7 mL dH_2O) and a 10% stacking layer. Western transfer buffer (1 L) consisted of the following: 3 g Tris base, 14.4 g glycine, 200 mL methanol, 10 mL 10% SDS, and

dH_2O to a final volume of 1 L.

After protein transfer, the membrane was blocked with 10% milk for 30 min, incubated with primary antibody in 5% milk for one hour, washed three times with Tris-Tween-Buffered Saline (TTBS), then incubated with HRP-conjugated secondary antibody in 5% milk for one hour, and finally washed three times with TTBS. TTBS (1 L) consisted of the following: 10 mL of 1M Tris, pH8.0, 30 mL of 5M NaCl, 500 µL of Tween20, and 959.5 dH_2O. Chemi-luminescence was used to detect HRP-conjugated antibodies and the substrate consisted of the following: 2 mL 1 M Tris pH 8.8, 44 µL p-coumaric acid (stocked at 0.15 g/ 10 mL DMSO), 100 µL luminol (stocked at 0.15 g/ 10 mL DMSO), 18 mL dH_2O, and 6 µL 30% H_2O_2. The luminol substrate solution (20.15 mL) was added to the membrane for one minute and then the membrane was exposed to film (Fujifilm Light Lab Systems 8" X 10" Cat# X-1002) for varying amounts of time to visualize the proteins of interest.

Antibodies and dilutions. Sheep anti-HIV Nef post 8 1:2000. Mouse anti-green fluorescent protein (Zymed 33-2600) 1:2000. Mouse anti-mono HA.11-16B12 (Covance MMS-101P) 1:2000. Rabbit anti-sheep HRP 1:10,000. Goat anti-mouse HRP 1:10,000.

Cell staining and flow cytometry. Transduced cells (500,000) or infected cells (100,000) were centrifuged at 1500 rpm for 5 minutes at 4°C (CR422 Jouan Tabletop Centrifuge; 770 rotor) in 5 mL polystyrene round-bottom tubes (BD Falcon) in a volume which varied depending on the concentration of the cells. Following centrifugation the supernatant was aspirated. For transduced cells, 5 µL of anti HLA-A1,11,26 (One Lambda, Inc., Cat. #0544HA) was then added for 20 min at 4°C. Cells were then washed twice with 2 mL FACS buffer (PBS supplemented with 4% FBS). FITC-conjugated goat anti-mouse IgM (2 µL) (Biosource AMI3608) was added for 20 min at 4°C. FACS Buffer (2 mL) was used to wash the cells (2X) and then mouse IgG (10 µg) (Sigma I5381) was added for 20 min at 4°C. FACS Buffer (2 mL) was used to wash the cells (2X) and mouse anti-CD4PE (7 µL) (Exalpha 0044) added for 20 min at 4°C). FACS Buffer (2 mL) was used to wash the cells (2X) and the cells were resuspended in 200 µL FACS buffer and 200 µL of 2% paraformaldehyde. Paraformaldehyde was prepared by adding 40 g of paraformaldehyde (PFA) into PBS (1L), heating to 56°C, cooling to 25°C, adjusting the pH to get PFA to go into solution, and then adjusting the final pH to 7.2-7.4. This 4% solution was then diluted in PBS to 2%. Cells were then analyzed on a Becton Dickinson Facs Calibur and Cell Quest software was used to analyze the data.

In vitro kinase assay. Whole cell lysate (600 μg) of transduced CEM T cells or transfected 293T cells were incubated in complete lysis buffer with 10 μL of sheep anti-HIV Nef antibody in a final volume of 800 μL overnight rotating at 4°C. Then 80 μL of a 50% protein A (Sigma P3391-5G) in PBS slurry were added to the lysate and rotated for 1.5 hours in at 4°C. Beads were centrifuged, washed twice with cold lysis buffer, once with 1 M magnesium chloride, and twice with kinase buffer and resuspended in 100 μL of room temperature kinase buffer. Kinase buffer consisted of 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, and 1% Triton X-100. γ^{32}P-ATP (μCi) (3 μL of 0.01 mCi/μL) was added to the beads for 10 min at room temperature. Reactions were stopped with 10 μL of 0.5 M EDTA and the beads centrifuged and supernatant removed. SDS buffer (100 μL of 3X) was added to the beads and vortexed briefly. This 3X buffer was comprised of 3 mL of 10% SDS, 3.33 mL glycerol, and 3.75 mL 1M Tris pH 6.8. The beads were centrifuged, the supernatant removed, and 5 μL of dye/reducing agent (1:4 saturated bromophenol blue: β-mercaptoethanol) added to the supernatant that was then incubated at 95°C for 5 min. Proteins were electrophoretically separated on a 10% polyacrylamide gel. The gel was stained with Coomassie blue, destained, and dried on a gel dryer for 1.5 hours at 80°C before exposure to a PhosphorImage screen overnight. Coomassie blue stain consisted of 0.25% Brilliant Blue R (Sigma B0149), 45% methanol, and 10% acetic acid. Phosphorylated proteins

were analyzed on a Cyclone Storage Phosphor System (Packard) and quantitated with Optiquant Image Analysis program and the gel was then exposed to film for autoradiography.

^{32}P-orthophosphate metabolic labeling. Three million Nef transduced HuT-78 or CEM cells were phosphate starved in phosphate free Dulbecco's Modified Eagle Medium (Gibco 11971) supplemented with 10% dialyzed fetal bovine serum (Gibco) for three hours. Cells were then pre-incubated in fresh phosphate free medium containing γ^{32}P -phosphoric acid at 0.4 mCi/mL (MP Biomedicals 64013). Cells were stimulated with 50ng/mL phorbol myristate acetate (Sigma P8139) for three hours. Cells were then put on ice and washed once with cold phosphate free media and once with cold PBS. Cells were then lysed in 500 μL complete lysis buffer, as described above.

The entire detergent soluble supernatant was immunoprecipitated with 10 μL sheep anti-HIV Nef post 8 antibody overnight at 4°C. Then 80 μL of a 50% protein A (Sigma P3391-5G) in PBS slurry were added to the lysate and rotated for 1.5 hours in at 4°C. Beads were centrifuged, washed twice with cold lysis buffer, once with 1 M magnesium chloride, and twice with kinase buffer. Proteins were eluted off the beads by addition of 100 μL 3X SDS loading buffer and heated at 95^{o}C for 5 minutes Proteins were then loaded onto a 10% acrylamide gel for

nine hours at 100 volts (900 volt hours) using the BioRad Power Pac300 (Model # 1655050) as a power source. The gel was stained with Coomassie blue, destained, and dried on a gel dryer for 1.5 hours at 80°C before exposure to a PhosphorImage screen overnight. Coomassie blue stain consisted of 0.25% Brilliant Blue R (Sigma B0149), 45% methanol, and 10% acetic acid. Phosphorylated proteins were analyzed on a Cyclone Storage Phosphor System (Packard) and quantitated with Optiquant Image Analysis program and the gel was then exposed to film for autoradiography.

Chapter 6: Investigation of Nef Phosphorylation

Introduction. Previous work has demonstrated HIV-1_{SF2} Nef is basally phosphorylated and this phosphorylation is increased 8-12 fold in response to phorbol myristate acetate (PMA) (Luo et al., 1997a). Based on phosphoamino acid analysis, HIV-1_{SF2} Nef has been shown to be primarily phosphorylated on serines. Treatment of cells with the PKA inhibitor, H-89 does not affect PMA induced phosphorylation however treatment with the PKC inhibitor, bisindolylmaleimide I blocks PMA induced phosphorylation (Luo et al., 1997a). Based on experiments investigating CD4 downregulation in HeLa.H4 cells, it was demonstrated that cells treated with PMA have increased CD4 downregulation suggesting a functional role for phosphorylation (Luo et al., 1997a).

Experimental objectives and questions to be addressed. Many outstanding questions remain regarding the HIV-1 Nef phosphorylation. First and foremost, it remains unknown which specific residues on Nef are phosphorylated. Also, the stoichiometry of basal and PMA-induced phosphorylation is unknown. Previous work in this lab as well as other reports in the literature suggest the phosphorylation may be protein kinase C mediated, however it still is unclear what

host cellular kinase(s) phosphorylate HIV-1_{SF2} Nef. The ultimate goal is to further understand the role of HIV-1_{SF2} Nef phosphorylation on viral pathogenesis.

The objective of this study was to identify the serine phosphorylation site(s) on HIV-1_{SF2} Nef, and to determine effect of phosphorylation on Nef cellular activities. One method to address this question would be to perform mass spectrometry analysis on basal and PMA treated Nef protein to identify putative phosphorylation sites as determined by post-translational modification analysis. However, the size of HIV-1_{SF2} Nef made it difficult to perform this analysis because the protein runs at the same molecular weight as the IgG light chain on polyacrylamide gels. Thus the protein could not be cut out of the gel for subsequent digestion and analysis. Moreover, the protein expression levels of phosphorylated Nef in transduced CEM T cells is not high enough to be purified for such analysis. In one study from the Skowronski group, Nef was purified for mass spectrometry analysis however this experiment used Jurkat cells stably expressing HIV-1_{NA7} that is tagged at the C-terminus (Janardhan et al., 2004). However, work in has demonstrated that C-terminal tags on Nef disrupts Nef association with other host cellular proteins (Raney et al., 2007). Thus, the approach undertaken in this study was to mutate all the highly conserved serines in HIV-1_{SF2} Nef and then to use these serine mutants for subsequent phosphorylation and functional analyses.

PMA increased HIV-1_{SF2} Nef phosphorylation in CEM T cells six-fold.

Human CEM T cells were transduced with the pLXSN control vector or HIV-1_{SF2} Nef. Cells were phosphate starved for three hours, then pre-equilibrated with γ^{32}P-phosphoric acid for another hour. Cells were left unstimulated or stimulated with 50ng/mL PMA for four hours. Based on previous time-course assays, four hours was determined to be the optimum stimulation time. Cells were then harvested and Nef protein was immunoprecipitated and proteins were separated by SDS-PAGE. Shown in Figure 6.1 is the autoradiography showing basally phosphorylated HIV-1_{SF2} Nef in lane 3 and PMA induced Nef phosphorylation in lane 4. PMA induced HIV-1_{SF2} Nef phosphorylation was six-fold greater than basal HIV-1_{SF2} Nef phosphorylation as determined by PhosphoImager quantitation. These data suggest that under these experimental conditions, HIV-1_{SF2} Nef is basally phosphorylated and this phosphorylation is increased by PMA.

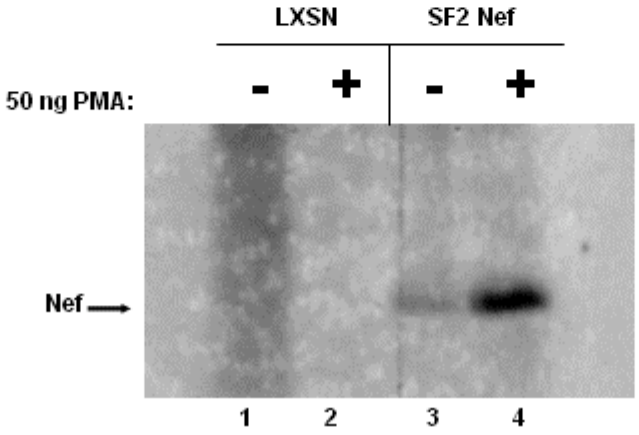

Figure 6.1 PMA increases HIV-1_{SF2} Nef phosphorylation in CEM T cells six-fold. Autoradiography of ^{32}P orthophosphate labeled HIV-1_{SF2} Nef immunoprecipitated from transduced CEM T cells. Lanes 1 and 2 are LXSN vector controls, lanes 3 and 4 are immunoprecipitates from LNefSNSF2 transduced cells. Cells from lanes 2 and 4 were treated with 50 ng/mL phorbol myristate acetate (PMA). Phosphorylation was quantitated using a PhosphorImager screen.

Mutagenesis strategy and sequence of highly conserved serines in HIV-1_{SF2} Nef. As previously mentioned, the mutagenesis strategy was to mutate the highly conserved serines to alanine for subsequent analysis. Shown in Figure 6.2 is the amino acid sequence for HIV-1_{SF2} Nef with the highly conserved serines highlighted in red. For these studies, highly conserved is defined as greater than 98% conserved. These residue are serines at positions 6, 34, 88, 103, and 187 (Figure 6.2). Each of these serine residues was mutated to alanine. However, it is known that serine 6 is necessary for the myristoylation signal (MGG*KW*S*K*R), therefore data on the S6A mutation would be subject to various interpretation. Serine 6 has also been proposed to be a putative protein kinase C phosphorylation site on Nef based on CD8-Nef truncation fusion proteins experiments (Wolf et al., 2008). One of the significant caveats of the work from Wolf et al. is that the impact of expressing Nef as the cytoplasmic part of a transmembrane protein is unknown.

1 MGGKW**S**KR**S**M GGW**S**AIRERM RRAEPRAEPA ADGVGAV**S**RD
41 LEKHGAIT**SS** NTAATNADCA WLEAQEEEEV GFPVRPQVPL
81 RPMTYKAALD I**S**HFLKEKGG LEGLIW**S**QRR QEILDLWIYH
121 TQGYFPDWQN YTPGPGIRYP LTFGWCFKLV PVEPEKVEEA
161 NEGENN**S**LLH PM**S**LHGMEDA EKEVLVWRFD **S**KLAFHHMAR
201 ELHPEYYKDC

Figure 6.2 Sequence of highly conserved serines in HIV-1_{SF2} Nef. The amino acid sequence of HIV-1_{SF2} Nef. Conservation as determined among 1643 HIV-1 subtype Nef sequences (O'Neill et al.). Serines with greater than 98% conservation are highlighted in red and underlined. The other lesser conserved serines are in black bold face.

To overcome the potential difficulties of mutating serine 6, a chimeric HIV-1_{SF2} Nef was constructed to maintain serine 6 and the myristoylation signal but alter the proposed PKC site. The myristoylation signal for SIV_{mac239} Nef is MGG*AI*S*M*R. Thus the HIV-1_{SF2} Nef myristoylation signal was replaced by the SIVmac239 signal to maintain the myristoylation signal while mutating the putative protein kinase C site as proposed by the Baur group (Wolf et al., 2008). The resultant mutant is referred to as HIV-1_{SF2} Nef mac myr which has the N-terminal sequence of MGGAISMR. This mutant was used along with HIV-1_{SF2} Nef S6A to determine whether serine 6 is in fact a protein kinase C site.

Mutation of highly conserved serines does not affect PMA induced phosphorylation. The aforementioned serine mutants were next used for phosphorylation analysis to determine which of these serines accounts for HIV-1_{SF2} Nef phosphorylation. Human HuT-78 cells were transduced with pLXSN control vector, HIV-1_{SF2} Nef and the S34A, S88A, S103A, and S187I serine mutants. Similar to the experiment in Figure 6.1, cells were either unstimulated or stimulated with PMA and Nef phosphorylation was quantitated as shown in the autoradiography in Figure 6.3A. Based on the quantitation, HIV-1_{SF2} Nef and the given mutants were all basally phosphorylated and the phosphorylation was increased in the presence of PMA. Apparent reductions in serine phosphorylation

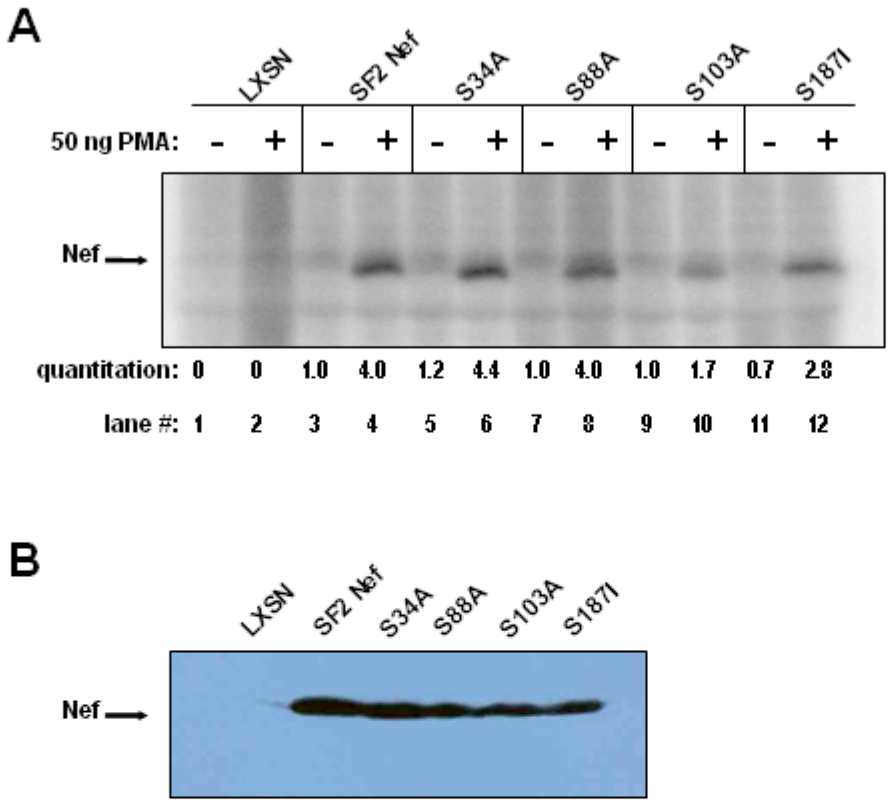

Figure 6.3 Mutation of highly conserved serines does not affect PMA induced phosphorylation. (A) Autoradiography of ^{32}P-orthophosphate labeled HIV-1_{SF2} Nef immunoprecipitated from transduced HuT-78 T cells. Cells were transduced with retroviral vectors for the indicated SF2 Nef serine mutants. Cells were either untreated or stimulated with 50 ng/mL PMA. Phosphorylation was quantitated using a PhosphorImager screen. (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

of S103A and S187A are unlikely to be the result of a loss in phosphorylation since similar reductions were observed in the protein expression levels (Figure 6.3B). These data suggest that all of these serines residues could be phosphorylated and therefore we could not ascertain which single residue would be primarily responsible for Nef phosphorylation. This suggested that HIV-1_{SF2} Nef is multiply phosphorylated.

Functional characterization of PAK-2 activation by HIV-1_{SF2} Nef serine mutants. Based on the previous phosphorylation experiment, it was concluded that the single serine residues at amino acid position 34, 88, 103 and 187 are not responsible for PMA enhanced HIV-1_{SF2} Nef phosphorylation. The ability of these Nef mutants to activate PAK-2 was investigated. Nef activation of PAK-2 was determined in these Nef serine mutants since the Nef/PAK-2 activation complex is present at low stoichiometry. Therefore phosphorylation of Nef within this complex may be below the level of detection by ^{32}P metabolic labeling methods. Human CEM T cells were transduced with HIV-1_{SF2} Nef and the indicated serine mutants and mac-myr Nefs and in vitro kinase assays were performed on the Nef immunoprecipitates. As shown in the autoradiography in Figure 6.4A, all the mutants activated PAK-2 to levels similar to HIV-1_{SF2} Nef based on adjustment to protein expression levels depicted in Figure 6.4B.

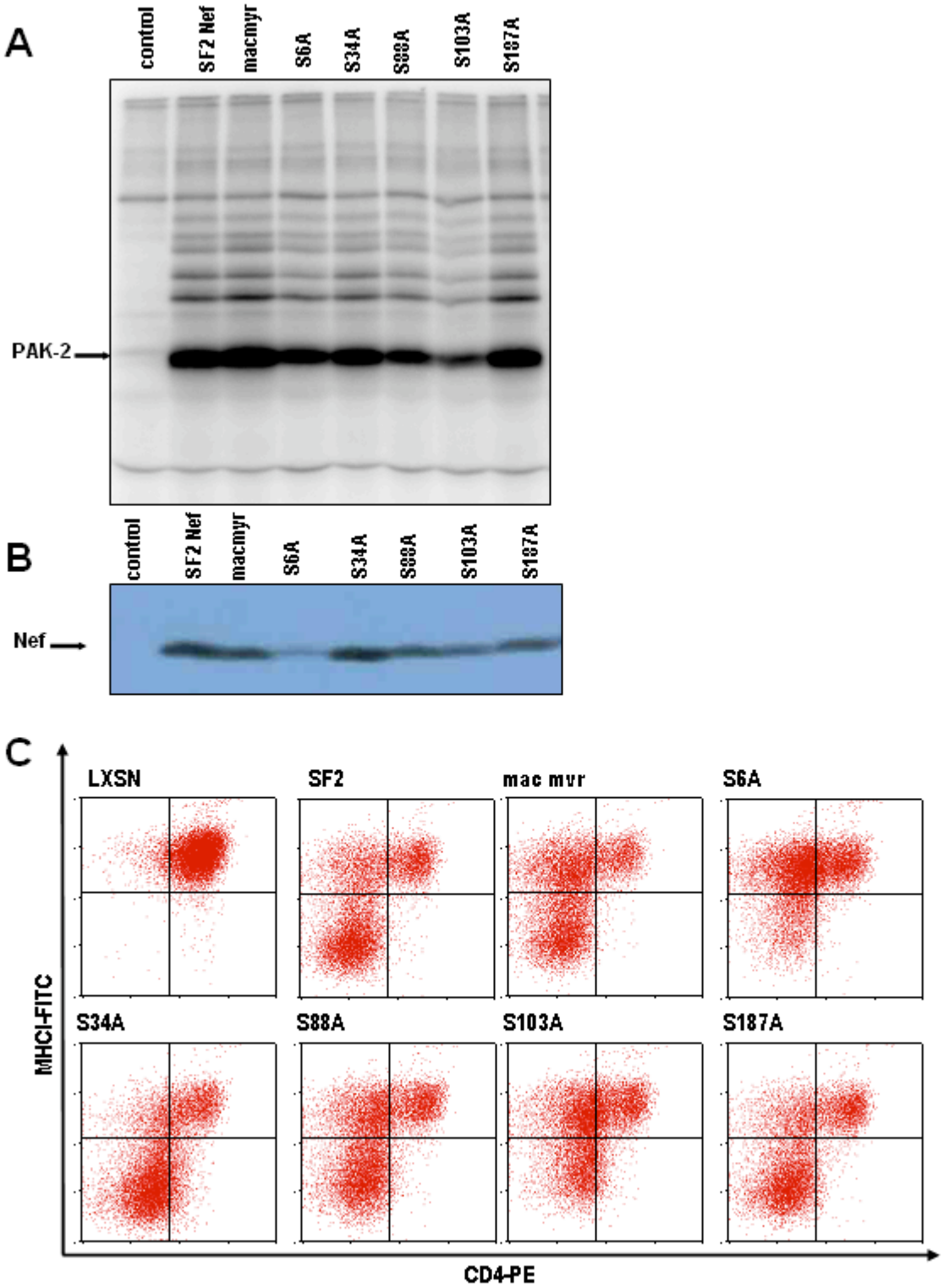

Figure 6.4 Functional characterization of HIV-1_{SF2} Nef serine mutants. CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective serine mutants. (A) Autoradiography of an in vitro PAK-2 kinase assay of SF2 Nef immunoprecipitates from transduced cells. (B) Western blot analysis demonstrating Nef protein expression from the transduced cells (C) Flow cytometry demonstrating cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis).

Therefore, these data suggest that the serine to alanine mutants and the mac-myr mutant have no affect on PAK-2 activation.

Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef serine mutants. In order to further evaluate the functional significance of these mutations, human CEM T cells were transduced with HIV-1_{SF2} Nef and the given serine mutants and mac-myr Nefs. Flow cytometry analysis was performed on these cells to assay for cell surface downregulation of CD4 and MHC-I. As shown in Figure 6.4C, all the mutants except for S6A downregulated CD4 and MHC-I to levels equivalent to HIV-1_{SF2} Nef. This suggests that these mutants are fully functional for CD4 and MHC-I downregulation. The HIV-1_{SF2} Nef S6A mutant downregulated CD4 but not MHC-I. This is attributed to the low levels of protein expression in the S6A mutant. As previously discussed, serine 6 is necessary for myristoylation of HIV-1_{SF2} Nef. As shown in the Western blot in Figure 6.4B, HIV-1_{SF2} Nef S6A is very poorly expressed as evinced by the faint band. This poor expression likely accounts for the defect in MHC-I downregulation because amount of MHC-I downregulation is highly sensitive to the cellular concentration of Nef, such that low or poor Nef protein expression will result in no MHC-I downregulation. Importantly, the HIV-1_{SF2} Nef mac-myr mutant was fully functional for CD4 and

MHC-I downregulation which would suggest that the putative protein kinase C site does not affect these Nef activities.

Conclusions. In summary, these data suggest that HIV-1_{SF2} Nef is multiply and redundantly phosphorylated. Not one serine residue alone dictates HIV-1_{SF2} Nef phosphorylation. Moreover this multiple phosphorylation occurs at basal as well as PMA induced levels. Given the nature of this phosphorylation, it is possible that phosphorylation occurs at non-conserved serines that could be allele specific. Therefore the functional significance of this phosphorylation will require further investigation. Moreover, identification of the kinase(s) responsible for this phosphorylation is still elusive. This study highlights the complexity of the potential role of phosphorylation on Nef function.

Chapter 7: Characterization of the Structural and Functional Determinants of Nef Activation of PAK-2

Introduction. Previous work has characterized several residues on HIV-1 Nef that are important for PAK-2 activation. Position 191 in subtype B HIV-1 Nef was first identified to be a key residue for PAK-2 activation based upon the observation that the primary isolate HIV-1_{233} Nef was defective for PAK-2 activation (Foster et al., 2001). HIV-1_{233} Nef has a isoleucine at position 191, however among 1643 subtype B HIV-1 Nef sequences, it is approximately 94% conserved to be phenylalanine at this position (O'Neill et al., 2006b). Other amino acids represented at 1% in the 1643 subtype B Nef sequences at this position are leucine, arginine, tyrosine, and isoleucine. Single mutations were introduced into HIV-1_{SF2} Nef to change phenylalanine at 191 to leucine, arginine and tyrosine. Each of these single mutants resulted in a complete loss of PAK-2 activation, demonstrating phenylalanine 191 is a key residue in regulating Nef activation of PAK-2 (O'Neill et al., 2006b). It is also important to note that while these experiments are focused on the ability of Nef to activate PAK-2, the mutations studied are specific for PAK-2 and do not affect the other functions of Nef. The four in vitro defined functions of Nef have been shown to be genetically independent of each other (Foster et al., 2001; O'Neill et al., 2006b).

It is not surprise that the drastic mutation of phenylalanine 191 to arginine ablated kinase activation. What was unexpected was the presence of arginine at this position in HIV-1 subtype E Nefs (O'Neill et al., 2006b). Among 1643 subtype B HIV-1 Nef sequences, 21 of the sequences are arginine at 191. Moreover, greater than 98% of subtype E HIV-1 Nef sequences have arginine at 191. Based on the initial experiment performed with subtype B HIV-1SF2 Nef, it could be postulated that the subtype E Nefs would be defective for PAK-2 activation as a result of the arginine substitution at 191. However this is not the case. In fact, five subtype E HIV-1 primary isolates were analyzed and found to be fully functional for PAK-2 activation and all five of these Nefs have arginine at position 191 (O'Neill et al., 2006b). These data suggested that as a result of having an arginine at position 191, there must be compensatory sites on subtype E Nef that allow for the preservation of PAK-2 activation.

The next step in this study was to look for "subtype-E like" residues among the subtype B HIV-1 Nef sequences. Specifically, the 21 subtype B sequences with arginine at 191 were analyzed (O'Neill et al., 2006b). Of these 21 sequences, 18 have a phenylalanine at position 89. This is in contrast to the majority of subtype B sequences which are 97% conserved to be histidine. These sequence analyses suggested that positions 89 and 191 are tightly coupled, thus Nef sequences have the combination of H89/F191 or F89/R191 are predicted to be

functional for PAK-2 activation. This observation further confirmed that the subtype E Nef sequences are greater than 98% conserved to be F89/R191 (O'Neill et al., 2006b). Thus, the phenylalanine at position 89 was hypothesized to be one of the compensatory sites on subtype E Nef to maintain PAK-2 activation.

In addition to positions 89 and 191, position 85 and 188 were also distinctly different in subtype E Nefs compared to subtype B Nefs. Point mutations were made at the sites in subtype HIV-1_{SF2} Nef to make a "subtype-E like" Nef. Each of these single "subtype-E like" mutations failed to restore PAK-2 activity, only the quadruple mutant L85F/H89F/K188A/F191R was able to completely restore PAK-2 activation (O'Neill et al., 2006b). This quadruple mutant is referred to as HIV-1_{SF2} Nef FFAR, or simply FFAR, to distinguish it from the residues present in HIV-1_{SF2} Nef (LHKF). Thus, FFAR is a fully functional subtype E PAK-2 interaction surface in a subtype B background. Of note, in no case were the other Nef functions lost, indicating that these sites are specifically involved in PAK-2 activation by Nef.

In a similar study, Agopian et al. also characterized this PAK-2 effector domain on Nef (Agopian et al., 2006). In addition to positions 85, 89, 188 and 191, Agopian et al. also analyzed position 187. The positions of these amino acids on the three-dimensional structure of Nef are shown in Figure 7.1. As noted above, subtype B HIV-1 Nef sequences are over 98% conserved serine at position

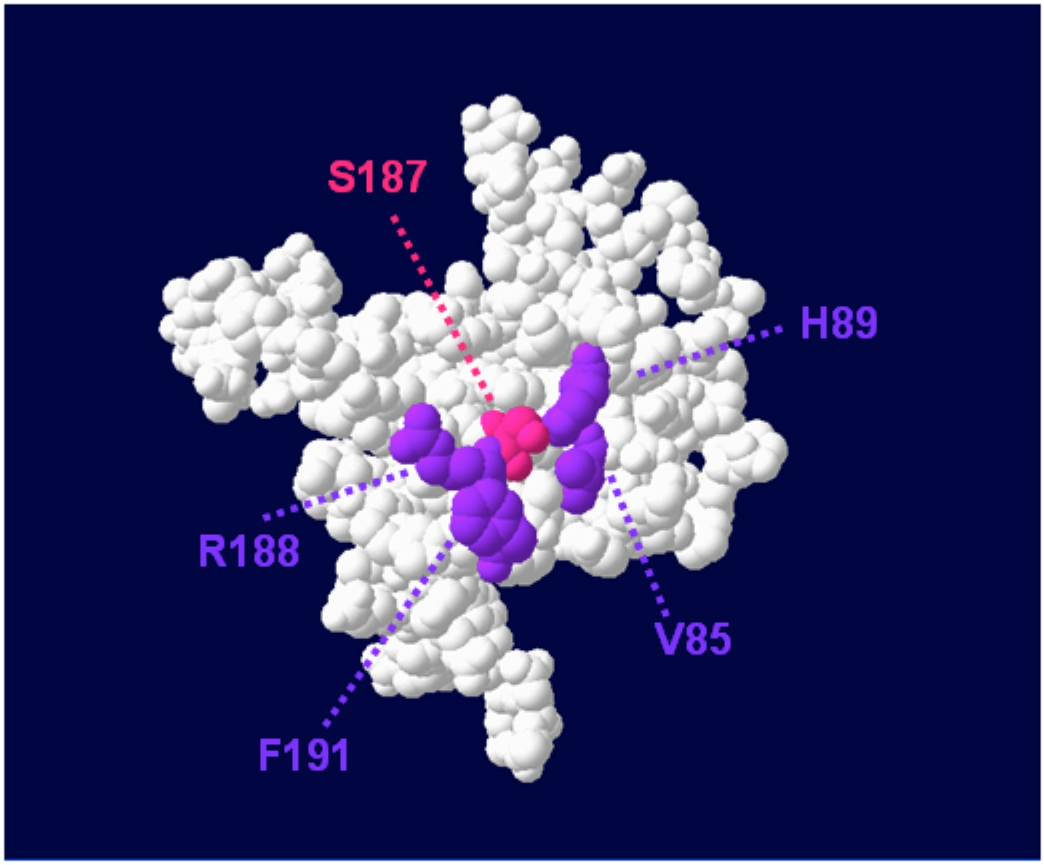

Figure 7.1 PAK-2 effector domain on HIV-1 subtype B Nef. HIV-1_{BH10} Nef, PBD:2Nef. The four residues characterized by O'Neill et al. are highlighted in purple, H89/V85/R188/F191. S187 is highlighted in pink. Together these residues constitute a hydrophobic domain on Nef that is critical for PAK-2 activation.

187. One interesting finding of Agopian et al. was that some Nef alleles induced many fold higher PAK-2 activation that HIV-1_{SF2} Nef (Agopian et al., 2006). Particularly active was the primary HIV-1 isolate MACS5C, herein referred to as 5C. The 5C Nef isolate came from a the frontal lobe brain tissue of a homosexual male patient enrolled in the Chicago Multicenter AIDS Cohort Study (Gorry et al., 2001). This 5C Nef has a hydrophobic isoleucine at position 187, which is contrary to the hydrophilic serine. Moreover, an in vitro kinase assay demonstrated that 5C Nef is hyperactive for PAK-2 activation compare to SF2 Nef (Agopian et al., 2006).

Based on the findings of these two independent studies (Agopian et al., 2006; O'Neill et al., 2006b) it is clear that positions 85, 89, 187, 188 and 191 function together to form an effector domain on Nef required for PAK-2 activation. The next important question to ask is how this domain varies between different subtypes of HIV-1. Previous work (O'Neill et al., 2006b) already characterized a subtype-E like structural variant FFAR, raising the question of whether there are other subtype specific structural variants? Also, can these studies be extended outside of HIV-1 or SIV? Based on the work of Agopian et al., what is the contribution of position 187 to PAK-2 activation? What is the effect of a single isoleucine amino acid substitution at 187 on PAK-2 activity? Moreover, what is the effect of a single isoleucine amino acid substitution at 187

in the context of the four other previously characterized residues? These are the questions that will be addressed in this thesis.

It was previously shown that a quadruple mutant in HIV-1_{SF2} Nef of L85F/H89F/K188H/F191H (herein referred to as FFHH) restores PAK-2 activation (O'Neill et al., 2006b) that was lost in the single HIV-1_{SF2} Nef F191H mutant. Therefore it appears that FFHH defines a third distinct effector domain for PAK-2 activation (LHKF, FFAR, and FFHH). Interestingly the hyperactive HIV-1_{5C} Nef is one of the rare subtype B Nefs that contains FFHH. Mutating isoleucine 187 in 5C Nef to the usual serine at this position yields a fully functional Nef that activated PAK-2 equivalently to HIV-1_{SF2} Nef, which is to say it loses its hyperactivity (Agopian et al., 2006).

Experimental objectives and questions to be addressed. The goal of this study was to further characterize subtype-specific alternative forms of Nef resulting in hyperactivation of PAK-2. In addition to subtype E, this study analyzed the PAK-2 interaction domain of a common subset of subtype C Nefs in a well-characterized subtype B SF2 Nef background. The presence of histidine at position 191 in subtype B Nef and the single substitution of phenylalanine 191 with histidine greatly diminishes PAK-2 activation (O'Neill et al., 2006b; Rauch et al., 2008). The role of residue 188 in SF2 Nef was investigated to determine if

mutations to arginine, histidine, serine, threonine, and leucine are fully or partially active.

In addition to positions 85, 89, 188 and 191, the contribution of substituting isoleucine at position 187 was also investigated. Substitution of isoleucine 187 in subtype B Nef (LHKF) was shown to completely abrogates PAK-2 activation. In stark contrast, isoleucine 187 in FFHH resulted in greater than 10-fold hyperactivation. Altogether, these sites represent a critical effector site on Nef for PAK-2 activation, and subtle changes in amino acid composition can dramatically affect kinase activation levels. This study highlights the importance for understanding the structural and sequence diversity of HIV-1 Nefs across different subtypes.

The K188L mutation in HIV-1_{SF2} Nef results in PAK-2 hyperactivation. Given the previous observation by Agopian et al that the HIV-1_{5C} Nef isolate is hyperactive, the next experiment was to investigate hyperactivity in the subtype B HIV-1_{SF2} Nef. Position 188 was previously characterized to be part of the PAK-2 effector site (O'Neill et al., 2006b). Point mutations were made at amino acid 188 in HIV-1_{SF2} Nef to alanine, arginine, histidine, serine, leucine and threonine. These amino acid substitutions are reflective of the amino acid composition at position 188 among 1643 subtype B HIV-1 Nef sequences.

As shown in the in vitro kinase assay in Figure 7.2A, amino acid substitution at position 188 to alanine, arginine, histidine, serine, and threonine had no effect on PAK-2 activity. Based on PhosphoImager quantitation, PAK-2 activity in these mutants was within in one-fold change, which herein will be considered to be normal kinase activation level. Surprisingly, the mutation of arginine to leucine in HIV-1_{SF2} Nef resulted in a 16-fold increase in PAK-2 hyperactivation. Such hyperactivation is comparable to the levels of PAK-2 activation seen in HIV-1_{5C} Nef. From these experiments, kinase hyperactivation is subsequently defined as greater than 5-fold activation relative to HIV-1_{SF2} Nef.

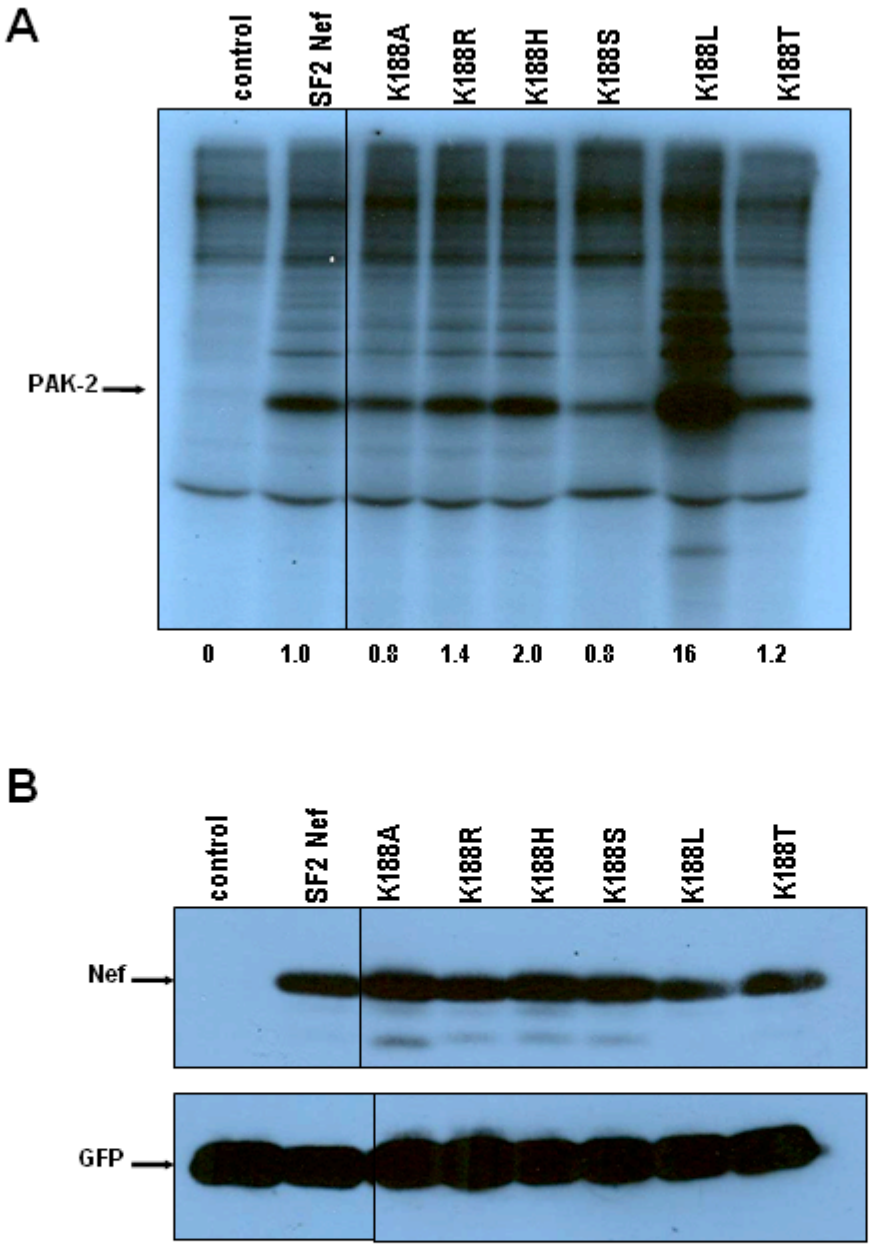

Figure 7.2 PAK-2 activation by HIV-1 Nef K188 mutants. (A) In vitro kinase assay on anti-Nef immunoprecipitates from transfected 293T cells. Autoradigraphy shows the autophosphorylated PAK-2 band indicated by the arrow. PhosphoImager quantitation relative to SF2 Nef is indicated below each lane (B) Western blot analysis demonstrating Nef protein expression from the transfected 293T cells and GFP protein expression as a transfection and loading control.

Functional characterization of CD4 and MHC-I downregulation in subtype B HIV-1_{SF2} Nef single mutants. The objective of this study was to investigate PAK-2 specific mutations on Nef. Thus prerequisite to defining PAK-2 functional changes, it is necessary to test the other in vitro cellular activities of Nef for the given mutants to ensure that these mutations are not causing global defects in the protein that could affect multiple functions of the protein. Similarly, protein expression levels must be confirmed. Before performing the PAK-2 analysis, the ability of the various mutants to induced CD4 and MHC-I downregulation was first assayed. The first mutants to be analyzed were the single mutations in HIV-1_{SF2} Nef K188L and S187I. HIV-1_{SF2} Nef K188L was already shown in 293T cells to be hyperactive for PAK-2 activation, as seen in Figure 7.2. HIV-1_{5C} Nef primary isolate that has an isoleucine at position 187 as opposed to the highly conserved serine.

Accordingly, human CEM T cells were transduced with the control vector, pLXSN or with pLXSN containing HIV-1_{SF2} Nef, HIV-1_{SF2} Nef K188L, or HIV-1_{SF2} Nef S187I. As shown in Figure 7.3B, Nef protein expression levels from cell lysates of the harvested CEM cells are comparable, thus these single mutations do not have any deleterious effects on protein expression. Cells from the same transduction were stained with antibodies against human CD4 and MHC-I, and flow cytometry analysis was performed to look at cell surface expression of CD4

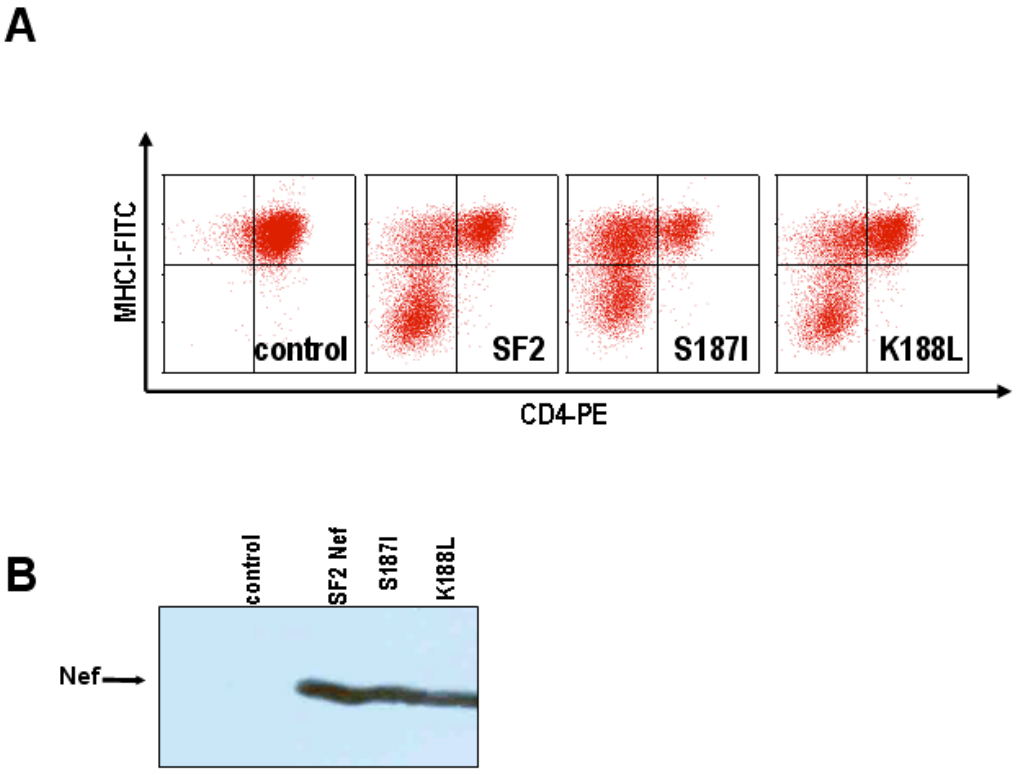

Figure 7.3 Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and the S187I and K188L mutants . (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective mutants. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

and MHC-I. As shown in Figure 7.3A, control cells had high expression of cell surface CD4 and MHC-I. Alternatively, cells expression SF2 Nef exhibited downregulation of CD4 and MHC-I. These levels of CD4 and MHC-I downregulation were the same with the S187I and K188L mutants. Thus, subtype B HIV-1_{SF2} Nef mutations S187I and K188L are functionally wildtype for CD4 and MHCI-I downregulation compared to HIV-1_{SF2} Nef.

Functional characterization of CD4 and MHC-I downregulation in HIV-1_{SF2} Nef subtype E-like mutants. Given the previous characterization of the FFAR structural variant (O'Neill et al., 2006b), the next question to ask is whether mutations at position 187 and 188 result in PAK-2 hyperactivation in the HIV-1_{SF2} Nef subtype E-like structure. Mutations were made in subtype B HIV-1_{SF2} Nef FFAR by site-directed mutagenesis to mutate serine 187 to isoleucine (FFIAR) and alanine 188 to leucine, serine and arginine (FFLR, FFSR, and FFRR). Also to test if positions 89 and 191 are tightly coupled in the FFAR domain, position 89 was mutated to histidine (FHAR). Based on previous work, it is hypothesized that uncoupling these two sites will result in a loss of function.

In order to make sure these mutations are specific for PAK-2, functional characterization of CD4 and MHC-I downregulation was first performed. As shown in Figure 7.4B, none of these mutations in the HIV-1_{SF2} Nef subtype E-like

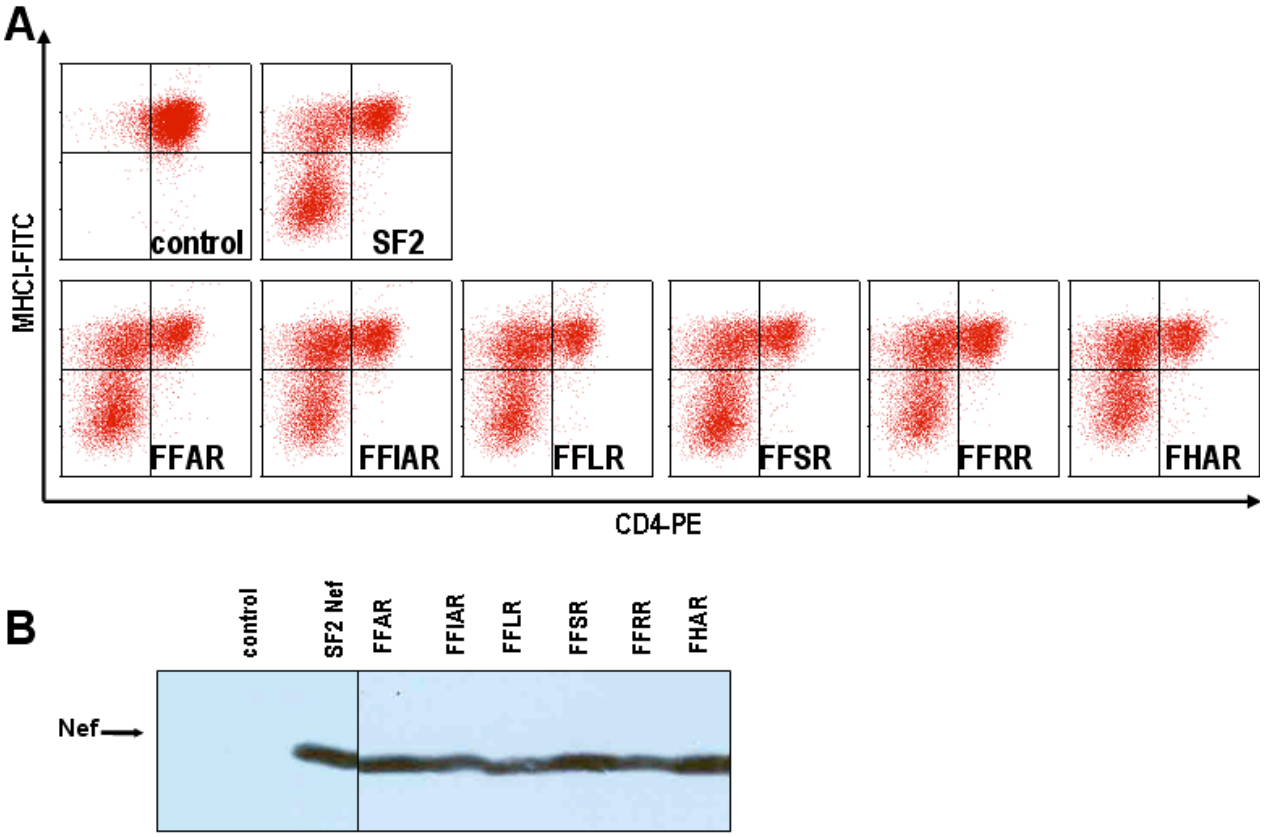

Figure 7.4 Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and the subtype E-like Nef variants . (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective mutants. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

structure have effects on protein expression levels. Flow cytometry to look at cell surface expression of CD4 and MHC-I of the transduced CEM T cells demonstrated that these mutations have no effect on CD4 or MHC-I downregulation, Figure 7.4A. These data show that mutations at position 187 and 188 have no effects on CD4 or MHC-I downregulation in the HIV-1_{SF2} Nef subtype E-like structure, confirming the suitability of these specific mutants for probing the structure function relations of PAK-2 activation as indicated below.

Functional characterization of CD4 and MHC-I downregulation in HIV-1_{SF2} Nef FFHH structural variant. In addition to the subtype E-like variant, structural variant found in HIV-1 subtype C Nefs has also been characterized (O'Neill et al., 2006a), the so-called FFHH. In contrast to previous results, O'Neill et al. found that HIV-1_{SF2} Nef FFHH was only 20% as active as HIV-1_{SF2} Nef, calling into question the validity of FFHH as unique PAK-2 activating effector domain. Moreover, the FFHH structural variant is similar to the HIV-1_{5C} Nef characterized by Agopian et al. where HIV-1_{5C} Nef is F85/F89/H188/H191. Agopian et al. demonstrated that HIV-1_{5C} Nef is hyperactive for PAK-2 activation. Thus, the next question to ask is whether mutations at position 187 and 188 in the FFHH subtype C- like structure results in either hypo- or hyperactivation of PAK-2 by Nef. To answer this, mutations were

constructed by site-directed mutagenesis at positions 187 and 188 of HIV-1_{SF2} Nef FFHH Nef. Serine 187 was mutated to isoleucine to generate the FFIHH mutant, which has the same amino acid composition at position 85, 89, 187, 188 and 191 as HIV-1_{5C} Nef. Position 188 was mutated to leucine or serine to generate the FFLH and FFSH mutants. Position 89 was also mutated back to histidine to uncouple positions 89 and 191 to make the FHHH mutant.

Functional characterization of CD4 and MHC-I downregulation in HIV-1_{SF2} Nef subtype c-like mutants was assayed by flow cytometry. Human CEM T cells were transduced to express the various HIV-1_{SF2} Nef subtype C-like mutants. As shown in the Western blot it Figure 7.5B, Nef protein expression levels in these subtype C-like mutants are equivalent to HIV-1_{SF2} Nef. Flow cytometry of CD4 and MHC-I cell surface expression in Figure 7.5A demonstrated that these HIV-1_{SF2} Nef mutants are also fully functional for CD4 and MHC-I downregulation.

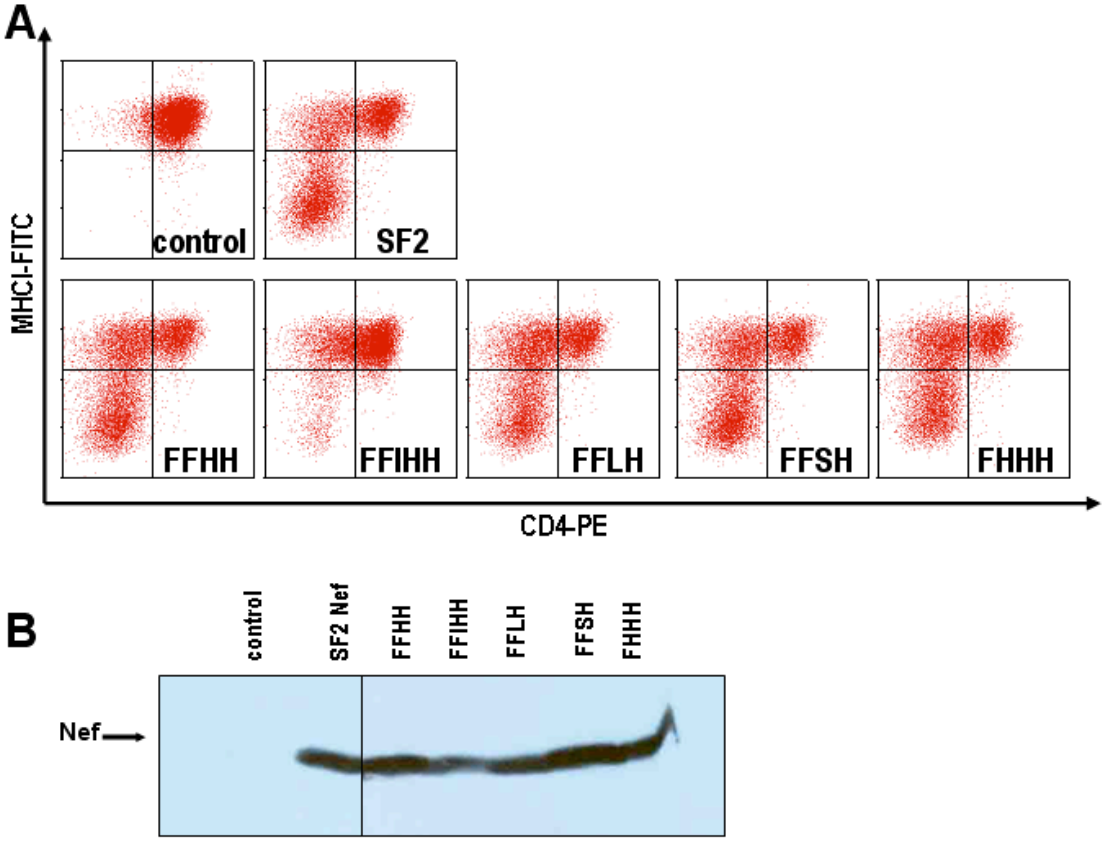

Figure 7.5 Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and the Nef FFHH variants . (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective mutants. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

PAK-2 activation by HIV-1_{SF2} Nef structural variants. The three subtype-specific HIV-1 Nef structural variants that will be compared are the subtype B-like (LHKF), subtype E-like (FFAR) and FFHH, found in subtype C HIV-1 Nef. Mutations were made at position 187 and 188 in these three structures to look for changes in PAK-2 activation levels. 293T cells were transfected with the indicated mutants, and harvested cell lysates were immunoprecipitated with anti-Nef antibodies. In vitro kinase assays were performed on the immunoprecipitated Nef complexes and the auto-phosphorylated PAK-2 band was visualized by autoradiography (Figure 7.6A). Western blot analyses in Figure 7.6B demonstrate similar Nef protein expression as well as GFP expression used as a transfection and loading control.

Single mutations of S187I and K188L in the subtype B-like Nef are shown in lanes 3 and 4, respectively. Compared to HIV-1_{SF2} Nef, S187 results in a complete loss of PAK-2 activation while K188L is hyperactive. This is quite remarkable give the close proximity of the two residues in the structure of this molecule (Figure 7.1). This is also surprising because HIV-1_{5C} Nef has an isoleucine at position 187 and yet it is hyperactive for PAK-2 activity.

Lanes 5 through 10 in Figure 7.6A correspond to the subtype E-like Nef structure. As previously characterized (O'Neill et al., 2006b), the subtype E-like structure in SF2 Nef (FFAR) has same level of PAK-2 activation as SF2 Nef. The

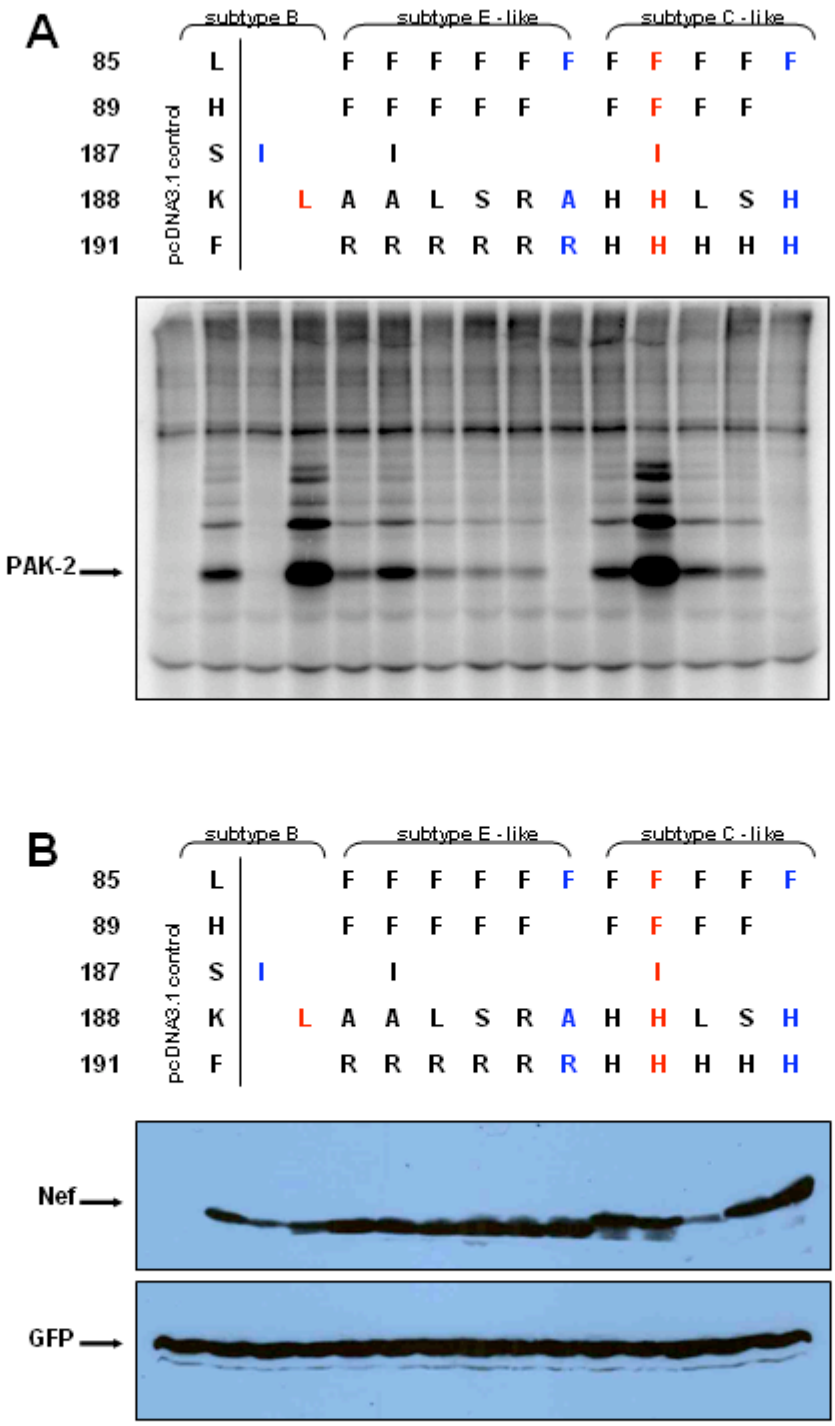

Figure 7.6 PAK-2 activation by HIV-1$_{SF2}$Nef structural variants. (A) In vitro kinase assay on anti-Nef immunoprecipitates from transfected 293T cells. Autoradigraphy shows the autophosphorylated PAK-2 band indicated by the arrow (B) Western blot analysis demonstrating Nef protein expression from the transfected 293T cells and GFP protein expression as a transfection and loading control.

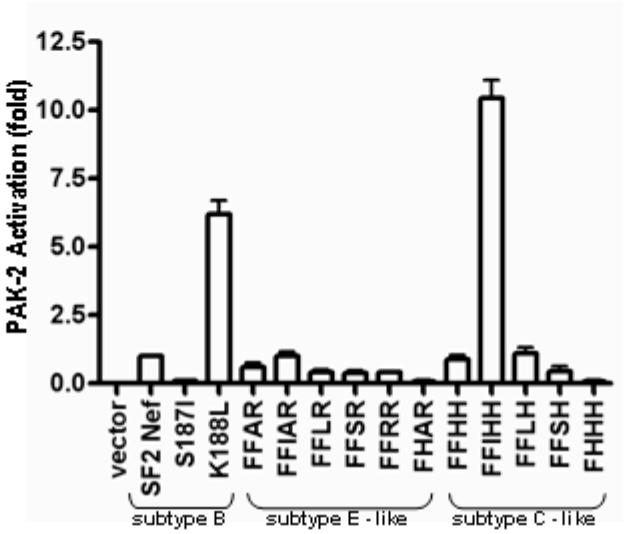

Figure 7.7 Quantitation of hypo and hyperactive PAK-2 activation by HIV-1_{SF2} Nef structural variants. Phosphoimager quantitation of PAK-2 autophosphorylated band from in vitro kinase assays as depicted in Figure 7.4 for one representative experiment. Quantitation based on four independent experiments.

single mutation of 187 to isoleucine (FFIAR) results in a modest increase in PAK-2 activation, however PhosphoImager quantitation (Figure 7.7) demonstrates that this change within one fold of SF2 Nef levels. Similarly mutations of position 188 in FFAR to leucine, serine and arginine (lanes 7 through 9) did not affect PAK-2 activity, such that the PAK-2 autophosphorylation levels again were still within one fold of SF2 Nef. Mutating position 89 in FFAR back to histidine completely ablates PAK-2 activation, which is expected given that F89 and R191 are tightly coupled.

Lanes 11 through 15 in Figure 7.6A correspond to the FFHH structure. Interestingly, the single mutation of 187 to isoleucine (FFIHH) results in PAK-2 hyperactivation. The FFIHH amino composition is the same as that seen in HIV-1_{5C} Nef. Thus isoleucine 187 confers PAK-2 hyperactivity in FFHH, while in subtype B Nef this results in a complete loss of PAK-2 activation, comparing lane 12 to lane 3. Similar to the previous analyses, position 188 in the FFHH subtype C-like variant was mutated to leucine and serine as shown in lanes 13 and 14. Neither mutation at position 188 in FFHH affected PAK-2 activation. Mutating position 89 in FFHH to histidine completely ablates PAK-2 activation, which is expected given that F89 and H191 are tightly coupled in the FFHH variant.

In summary, single amino acid changes can dictate PAK-2 hypo- or hyperactivation in a subtype dependent manner. Nef defective for PAK-2

activation is defined as less than or equal to 0.2 fold of HIV-1_{SF2} Nef. As shown in Figure 7.7, HIV-1_{SF2} Nef S187I, FHAR and FHHH are all PAK-2 activity defective. FHAR and FHHH were expected to be defective as a result of uncoupling position 89 and 191. HIV-1_{SF2} Nef S187I is also completely defective for PAK-2 activation however the isoleucine substitution in the FFHH variant results in hyperactivation and has no affect on the subtype E-like structure (FFAR). These subtype specific substitutions are further demonstrated at position 188. The single leucine substitutions at 188 in subtype B Nef results in hyperactivation, however leucine 188 in the subtype E or C-like structures has no effect on activity.

The effect of Nef-mediated PAK-2 hyperactivation on merlin phosphorylation. The next important question to ask is what is the downstream functional consequence of PAK-2 hyperactivation? Previous work has demonstrated that PAK-2 activated by Nef can phosphorylate merlin (Moesin, Ezrin, and Radixin-Like protein) on serine 518. In the absence of Nef, merlin exists in the hyper and hypophosphorylated state. The mutation of serine 518 to alanine prevents merlin phosphorylation such that only the hypophosphorylated species is present, as shown in lanes 2 and 4 of Figure 7.8A. However in cells expressing Nef, PAK-2 is activated such that there is more of the

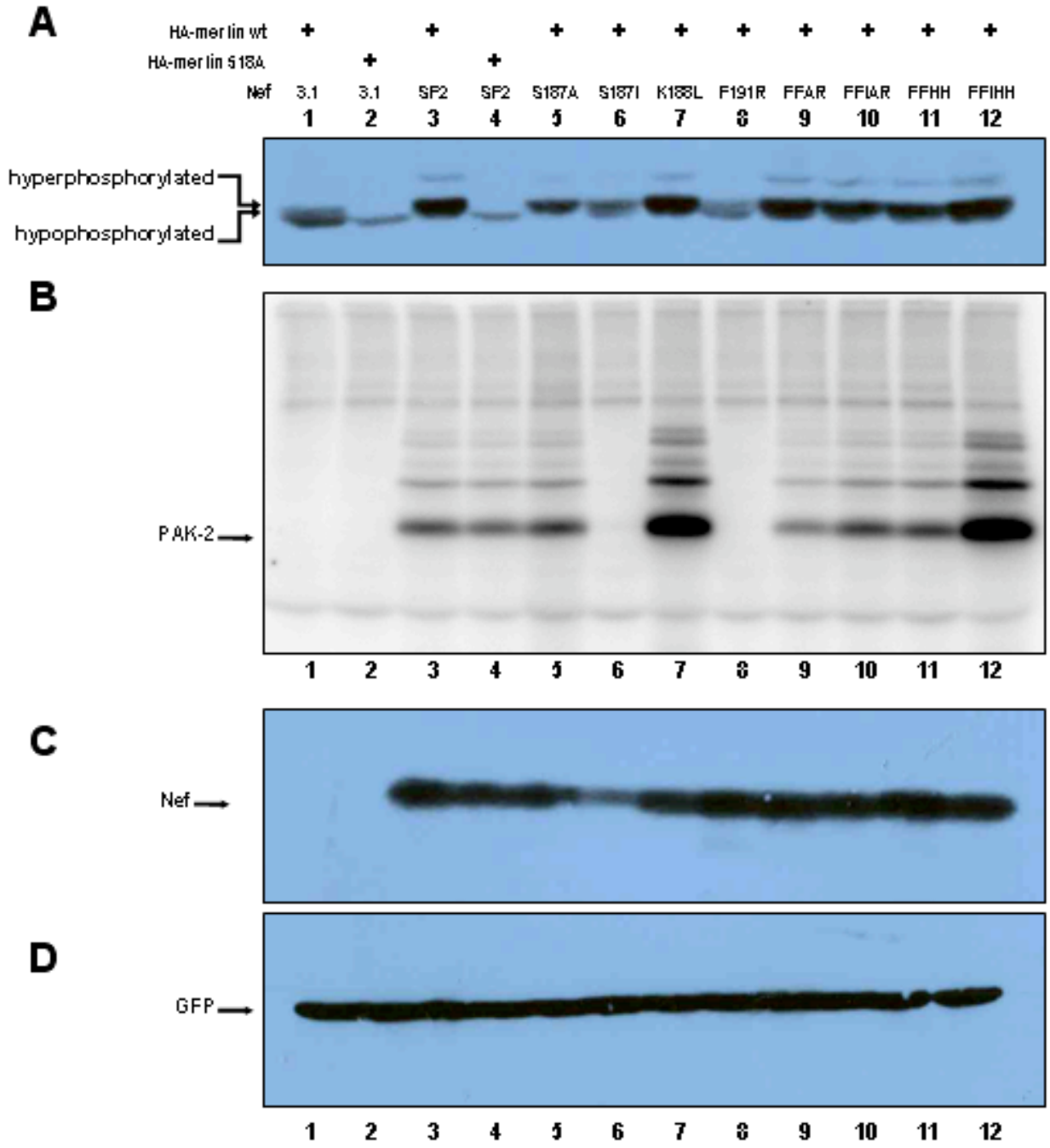

Figure 7.8 Merlin phosphorylation by PAK-2 mediated by HIV-1_{gr2} Nef. 293T cells were transfected with HA-merlin or HA-merlin 518A and the listed Nef constructs. Whole cell lysates were used for the three Western blots shown (A) Anti-HA Western blot showing the hyper and hypophosphorylated species of merlin as indicated by the arrows. (B) Autoradiography of an in vitro kinase assay of the same cell lysates. The autophosphorylated PAK-2 band is indicated with the arrow. (C) Western blot analysis demonstrating Nef protein expression (D) Ant-GFP Western blot as a transfection and loading control.

hyperphosphorylated merlin, comparing lanes 3 and 1 in Figure 7.8A. To this end, merlin phosphorylation levels were investigated in the three different HIV-1 structural variants. As shown in lanes 4 through 10, both the hyper and hypophosphorylated species of merlin were observed in cells expressing the different Nef. These data indicate that Nef activation in PAK-2 in these cells results in merlin phosphorylation.

Analysis of HIV-1 alternative structures does not extend to SIV. Thus far, investigation of the PAK-2 effector site on Nef has been limited to HIV-1. It would be therefore important to ask whether these analyses can be extended to other primate lentiviruses, specifically simian immunodeficiency virus (SIV) Nef. Briefly, HIV-1 in humans originated from SIV in chimpanzees and HIV-2 originated from SIV in sooty mangabeys such that SIV Nef is closely related to HIV-1 Nef. HIV-1 and SIV Nef share nearly all the same function, including PAK-2 activation (Kirchhoff et al., 2008). It is important to note that SIV_{mac239} Nef is larger than $HIV\text{-}1_{SF2}$ Nef, 263 amino acids compared to 210 amino acids, respectively. Both SIV_{mac239} Nef and $HIV\text{-}1_{SF2}$ Nef have been well characterized in their ability to activate PAK-2 (Arora et al., 2000; Sawai et al., 1996). Given the conservation of this activity, it is possible that SIV_{mac239} Nef also has an alternative structure regulating PAK-2 activation. To test this hypothesis, point

mutations were made in HIV-1_{SF2} Nef at positions 85, 89, 188, and 191 to corresponding SIV_{mac239} Nef amino acids to generate the in HIV-1_{SF2} Nef SIV-like L85I/H89/K188T/F191Y mutant, herein referred to as IHTY.

Human CEM T cells were transduced with control pLXSN vector, SF2 Nef, or HIV-1_{SF2} Nef IHTY. As shown in the Western blot in Figure 7.9C, there are similar levels of Nef protein expression in these transduced cells. Cells were subjected to flow cytometry analysis to assay cell surface expression of CD4 and MHC-I. The HIV-1_{SF2} Nef IHTY mutant downregulated CD4 and MHC-I as well as HIV-1_{SF2} Nef, suggesting that these SIV-like mutations in HIV-1_{SF2} Nef do not have deleterious effects on CD4 or MHC-I downregulation.

Cell lysates from the same transduced CEM cells were immunoprecipitated to perform in an vitro kinase assay. As shown in Figure 7.9B, HIV-1_{SF2} Nef SIV-like IHTY mutant does not activate PAK-2, indicating that the SIV-like residues in HIV-1_{SF2} Nef do not confer a functional PAK-2 effector on HIV-1_{SF2} Nef. These data suggest that PAK-2 activation by SIV_{mac239} Nef is mediated by residues that are not equivalent to the four previously characterized residues in HIV-1 Nef.

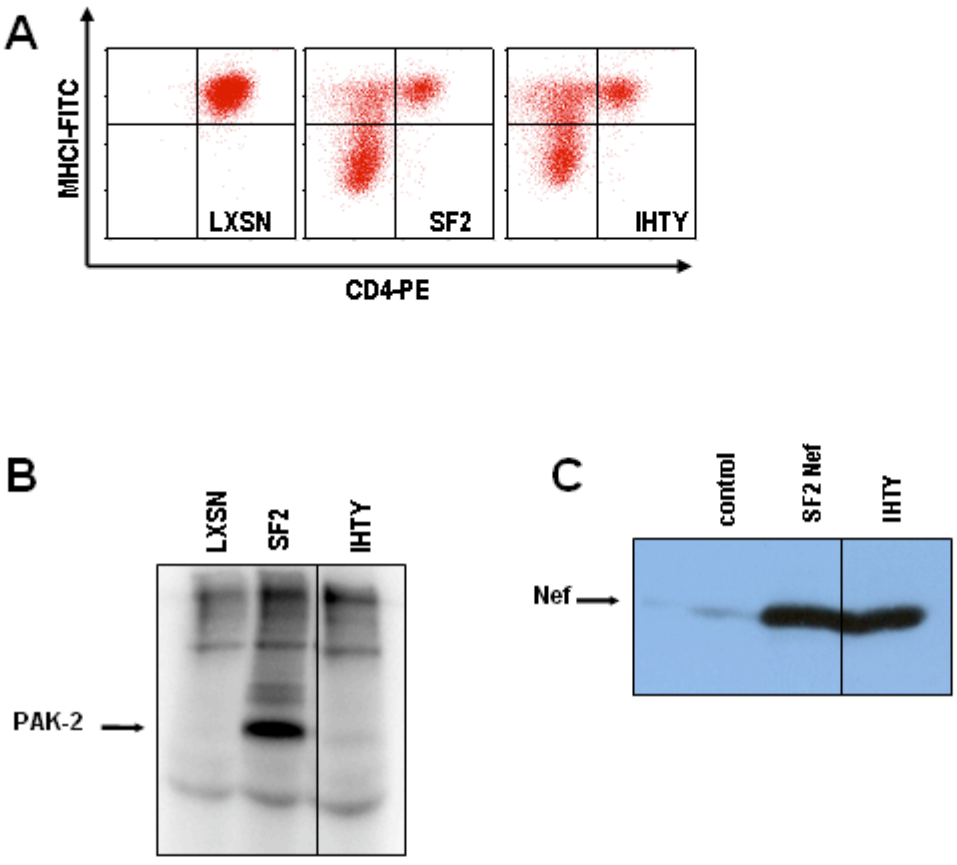

Figure 7.9 Analysis of HIV-1 alternative structural variants does not extend to SIV. (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and SIV variant L85I/H89/K188T/F191Y. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) In vitro kinase assay on anti-Nef immunoprecipitates from the transduced CEM cells (C) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

Juxtaposed functional domains on HIV-1 subtype B Nef. Altogether, the data suggest amino acid position 85, 89, 187, 188, and 191 on HIV-1_{SF2} Nef constitute a critical PAK-2 effector domain on Nef. The domain is specific for PAK-2 activation. These five positions form a core domain on Nef, as depicted in Figure 7.1. However given the multifunctional nature of Nef, different regions on the protein are also thought to regulate different cellular functions. The N-terminal flexible arm of Nef is thought to be involved in MHC-I downregulation (Baugh et al., 2008). The acid cluster $_{62}$EEEE$_{65}$ is highlighted in red in Figure 7.10. The next three residues highlighted in orange are valine 66, glycine 67, and phenylalanine 68. These residues are over 99% conserved among subtype B Nef sequences, suggesting some functional importance however the specific function of these three residues remains unknown.

HIV-1 contains a highly conserved polyproline tract, depicted in Figure 7.10 in green and yellow. This $_{69}$PVRPQVPLRP$_{78}$ is a canonical class II SH3 binding domain, and it is thought that this region of Nef mediates binding to host cellular SH3 containing protein(s). It has been previously postulated that an SH3 binding protein is required for PAK-2 activation based on the P72A/P75A double mutant (Manninen et al., 1998). The work presented here demonstrates that Nef activation of PAK-2 is mediated by residues in the Nef hydrophobic core, depicted in purple and pink in Figure 7.10. This further demonstrates that there are

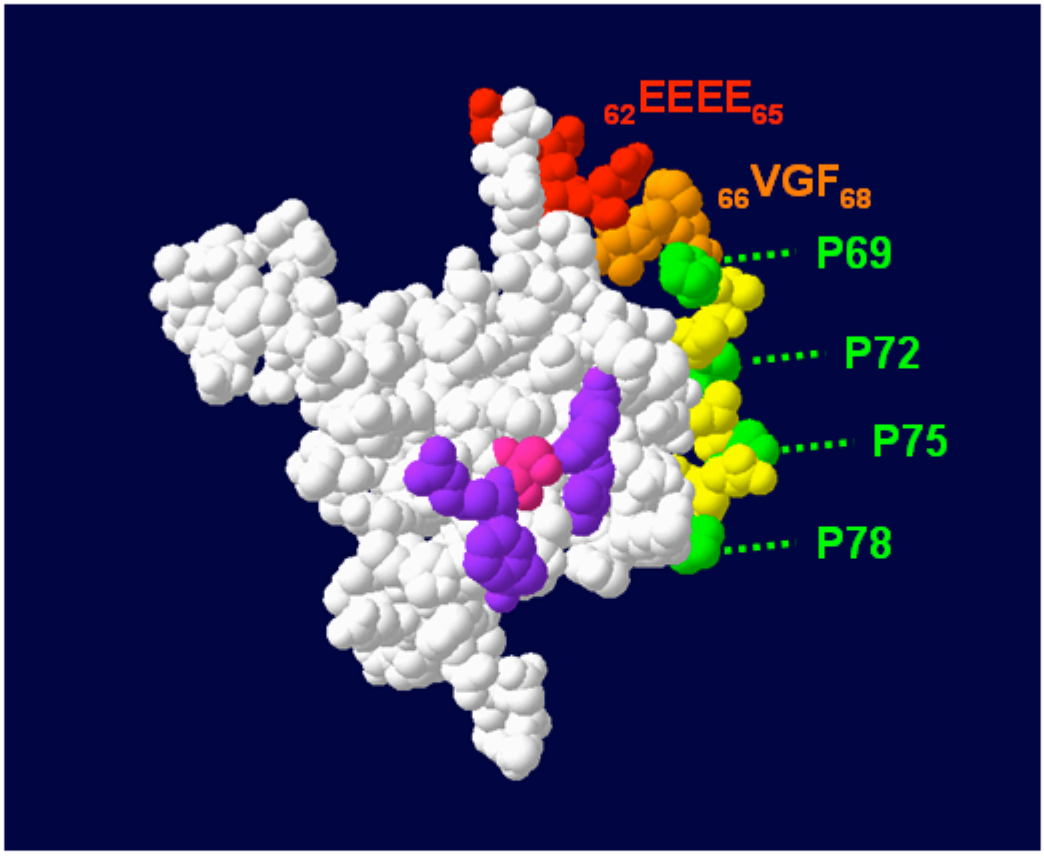

Figure 7.10 Juxtaposed functional domains on HIV-1 subtype B Nef. HIV-1_{BH10} Nef, PBD:2Nef. The four residues characterized by O'Neill et al. are shown in purple, H89/V85/R188/F191. S187 is indicated in pink. The acidic domain $_{62}$EEEE$_{65}$ is indicated in red. The $_{66}$VGF$_{68}$ linker region is shown in orange. The polyproline helix is in yellow with the individual proline residues in green.

multiple functional domains on Nef. Based on the data presented here, it is hypothesized that this core effector domain on Nef constituted by residues 85, 89, 187, and 191 specifically regulates PAK-2 activation by Nef. It is also hypothesized that the regulatory regions towards the N-terminus of HIV-1 Nef regulate multiple functions of Nef such that mutations in the region will result in multiple defects in Nef cellular activity. Specifically, experiments were conducted to first examine of the role of the $_{66}$VGF$_{68}$ linker on function and next the role of the role of the polyproline tract in HIV-1$_{SF2}$ Nef.

Functional characterization of CD4 and MHC-I downregulation by HIV-1$_{SF2}$ Nef and the $_{66}$VGF$_{68}$ linker region mutants. As previously mentioned, $_{66}$VGF$_{68}$ is highly conserved among subtype B HIV-1 Nef sequences suggesting that these residues may be important for function. These residues also form a linker region between the highly conserved Nef acidic cluster and the polyproline helix. Mutations were made in HIV-1$_{SF2}$ Nef at these sites to generate V66A, G67A, and F68A. These HIV-1$_{SF2}$ Nef linker region mutant constructs were transduced into CEM T cells for Western blot and flow cytometry analysis. As shown in Figure 7.11B, there are similar levels of Nef protein expression in all three mutants compared to HIV-1$_{SF2}$ Nef. Flow cytometry analysis to assay for cell surface expression of CD4 and MHC-I shown in Figure 7.11A demonstrated

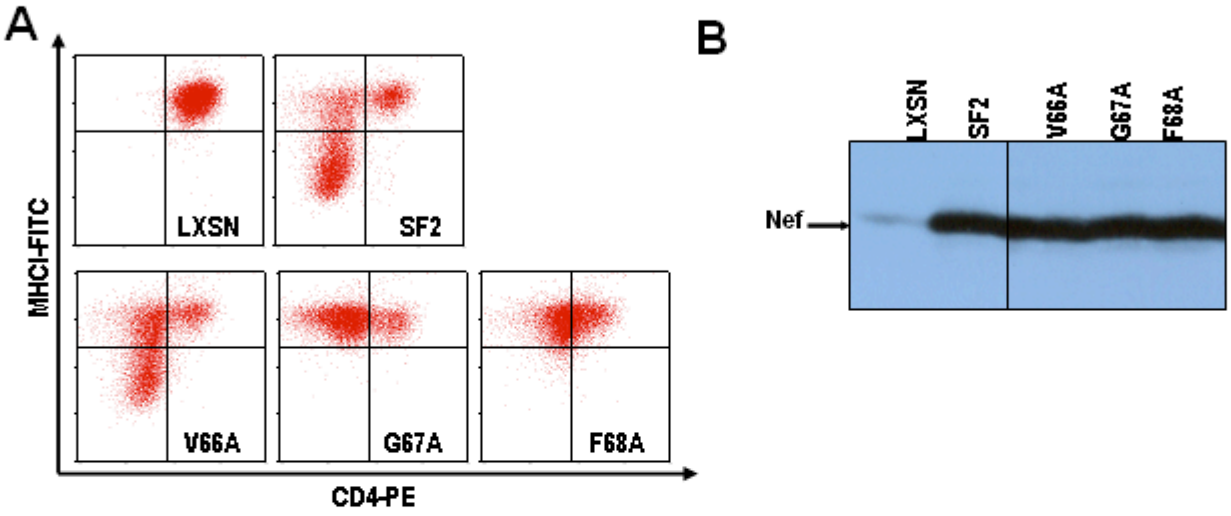

Figure 7.11 Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and VGF linker region mutants. (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective mutants. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

that the V66A mutation had no deleterious effect on CD4 and MHC-I downregulation compared to HIV-1_{SF2} Nef. However, the G67A and F68A mutants were completely defective for MHC-I downregulation, although they are both functional for CD4 downregulation.

Functional characterization of PAK-2 activation by HIV-1_{SF2} Nef and the $_{66}$VGF$_{68}$ linker region mutants. PAK-2 activation by the linker region mutants was next assessed by performing in vitro kinase assays on the same transduced cells from Figure 7.11. As shown in the autoradiography in Figure 7.12A, the V66A mutants have normal levels of PAK-2 activation compared to HIV-1_{SF2} Nef. However, the G67A and F68A mutants were completely defective in PAK-2 activation. These defects are not due to poor protein expression because there are equivalent amounts of Nef protein in these cells as shown in Figure 7.12B. Thus, it can be concluded that this region of Nef is involved in both MHC-I downregulation and PAK-2 activation.

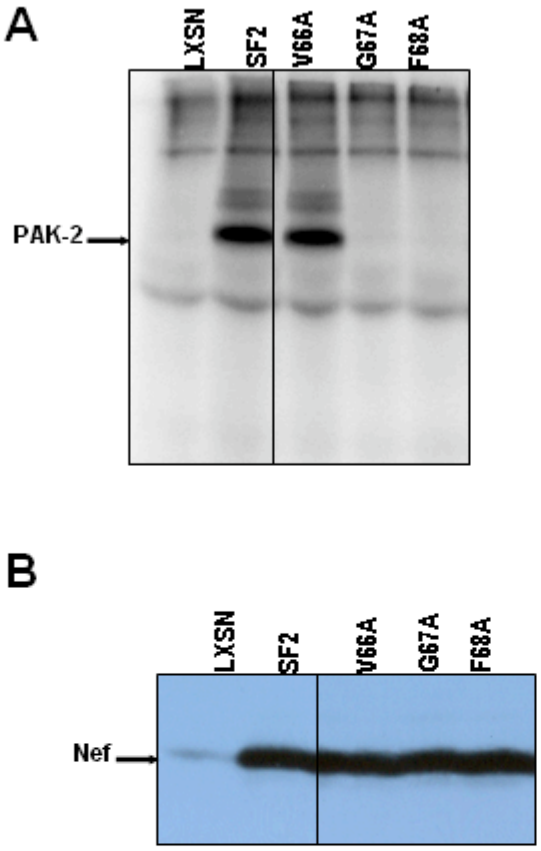

Figure 7.12 Functional characterization of PAK-2 activation by HIV-1_{SF2} Nef and VGF linker region mutants. (A) In vitro kinase assay of anti-Nef immunoprecipitates from transduced CEM T cells (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A

Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and the polyproline helix mutants. The objective of these experiments was to determine whether mutations in the HIV-1_{SF2} Nef polyproline helix affect CD4 and MHC-I downregulation. Accordingly, the following conservative point mutations were made by site directed mutagenesis in HIV-1_{SF2} Nef: Q73R, V74I, L76V, and R77K. The HIV-1_{SF2} Nef P72G and P75G mutations were also made. Previously, the P72A/P75A double mutant has been shown to disrupt MHC-I downregulation. These HIV-1_{SF2} Nef constructs were used for transduction of CEM T cells to express the various Nef mutants. As shown in the Western blot in Figure 7.13B, these single mutations have no deleterious effects on protein expression such that equal Nef protein levels were found in the transduced Nef cells. Cells were subjected to flow cytometry analysis to assay cell surface expression of CD4 and MHC-I. Compared to HIV-1_{SF2} Nef, all the polyproline helix mutants downregulated CD4. However there were modest defects in MHC-I downregulation in the P72G and P75G mutations, suggesting that these two proline residues are necessary for optimal MHC-I downregulation.

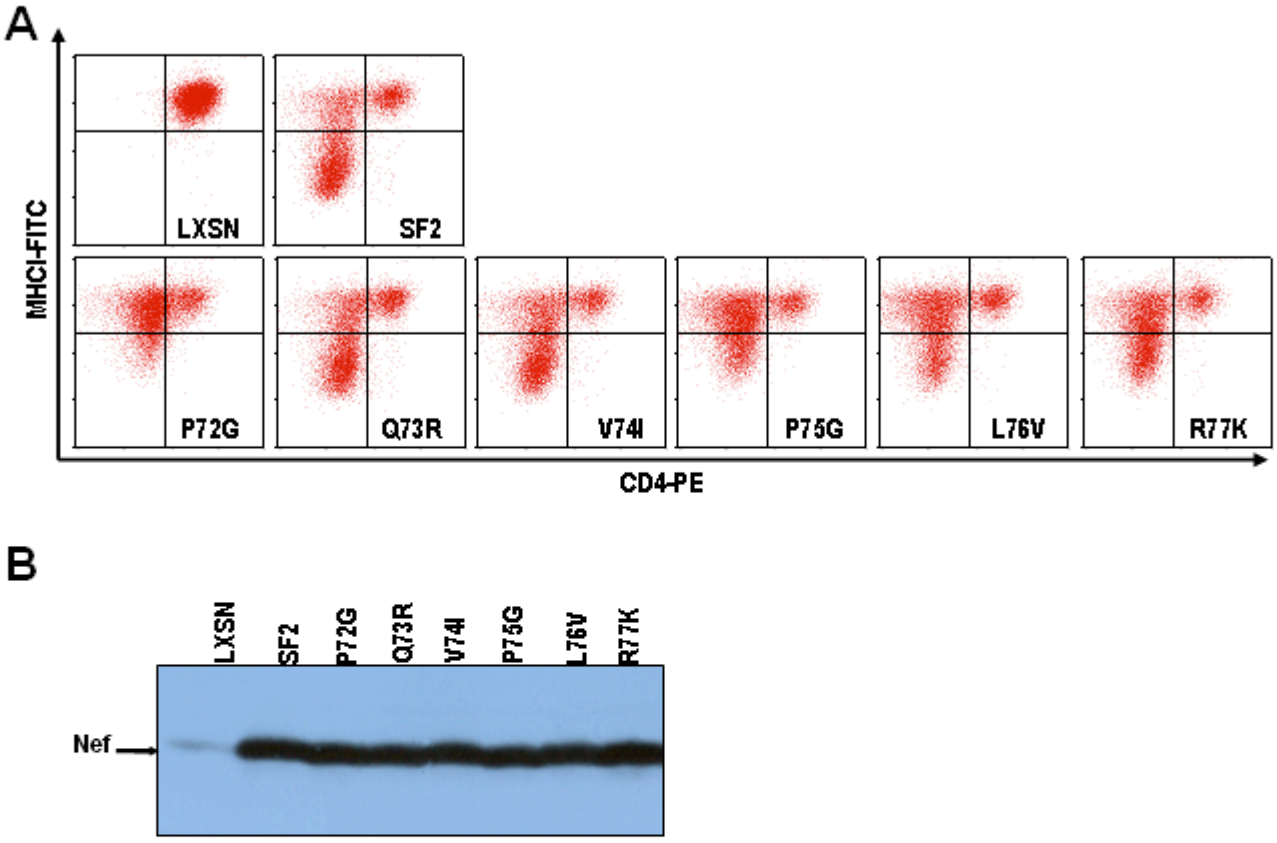

Figure 7.13 Functional characterization of CD4 and MHC-I downregulation by HIV-1_{SF2} Nef and the polyproline helix mutants. (A) Flow cytometry of CEM T cells transduced with LXSN control vector, HIV-1_{SF2} Nef, and the respective mutants. Cell surface expression of CD4 labeled with PE (x-axis) and MHC-I labeled with FITC (y-axis). (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A.

Functional characterization of PAK-2 activation by HIV-1_{SF2} Nef and the polyproline helix mutants. The same transduced CEM T cells from Figure 7.13 were also used for in vitro kinase assays to evaluate PAK-2 activation by HIV-1_{SF2} Nef and the given polyproline helix mutants. As shown in the autoradiography in Figure 7.14A, mutation of the polyproline helix cause significant defects in PAK-2 activation. Only the V74I mutant was able to activate PAK-2 to HIV-1_{SF2} Nef levels. There is only partial PAK-2 activation in the L76V mutant, while PAK-2 activation is completely lost in the P72G, Q73R, P75G, and R77K mutants. These data support the previous conclusion that this region of Nef is important for PAK-2 activation and serves as an SH3 binding domain.

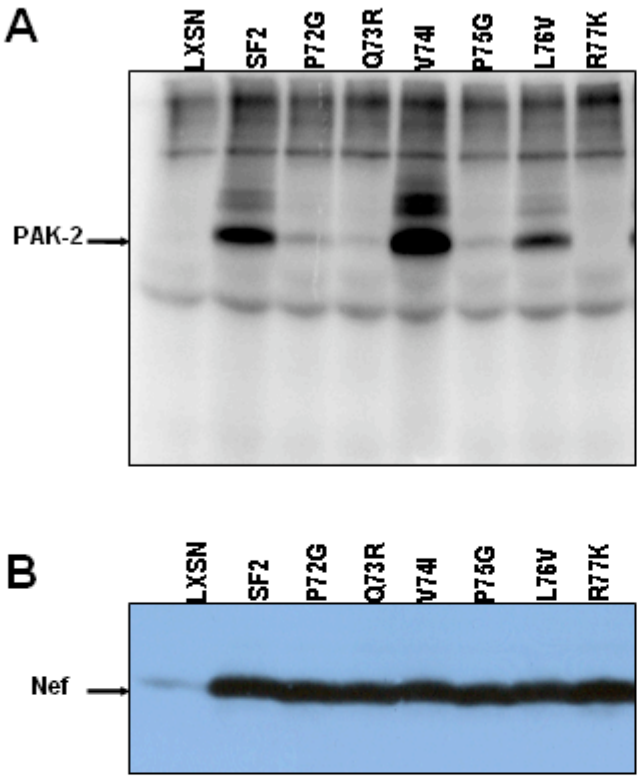

Figure 7.14 Functional characterization of PAK-2 activation by HIV-1_{SF2} Nef and the polyproline helix mutants. (A) In vitro kinase assay of anti-Nef immunoprecipitates from transduced CEM T cells (B) Western blot analysis demonstrating Nef protein expression from the transduced cells in part A

Conclusions. These studies have characterized the ability of single amino acid changes to dictate hyperactivation of PAK-2 by HIV-1 Nef. Previously published work has identified a unique PAK-2 effector site on HIV-1 Nef that is constituted by four amino acid position 85, 89, 188, and 191. These studies have been extended to investigate the role of position 187 in this structure of Nef. Hyperactivation of subtype B HIV-1_{SF2} Nef occurs upon mutation of position 188 from lysine to leucine. However this amino acid substitution does not result in PAK-2 hyperactivation in the subtype E or subtype C-like structural variants. Similarly, the substitution of isoleucine for serine at position 187 completely ablates PAK-2 activation subtype B HIV-1 Nef yet this same substitution in the FFHH structural variant results in PAK-2 hyperactivation.

This unique PAK-2 effector site at the hydrophobic core of Nef regulates function in a different manner than the N-terminal region of Nef. The three main functional domains at the N-terminal region of Nef of interest are the acidic cluster, the linker region, and the polyproline helix. Mutations of these sites result in multiple defects in Nef function, specifically affected MHC-I downregulation and PAK-2 activation. These data highlight the multifunctional nature of Nef and its ability to regulate different cellular activities via different structural and functional domains of the protein.

Chapter 8: Conclusions and Recommendations for Nef Phosphorylation

HIV-1_{SF2} Nef is multiply and redundantly phosphorylated. HIV-1_{SF2} Nef phosphorylation occurs at basal levels, and this phosphorylation is greatly increased presence of the protein kinase C activator phorbol myristate acetate (PMA) and inhibited by H89 (Luo et al., 1997a). This finding indicates that HIV-1_{SF2} Nef phosphorylation is increased as a function of PKC activation. Moreover, treatment of cells with PMA and bisindolylmaleimide I, a PKC inhibitor blocks PMA induced phosphorylation. One of the objectives of this thesis was to investigate the significance of putative serine phosphorylation sites as PKC substrate sites on HIV-1_{SF2} Nef.

Serine 6 of HIV-1_{SF2} Nef is not a PKC site. Work from the Baur group has proposed HIV-1_{SF2} Nef serine 6 (MGGKW***S***KRS) is phosphorylated by PKCδ and PKCθ in vitro. The key caveat to this experiment was that the authors demonstrated this phosphorylation with a chimeric membrane spanning fusion protein of CD8 and the first 35 amino acids of HIV-1_{SF2} Nef (CN.35), but not with full length Nef. In this study, we investigated the relevance of serine 6 in phosphorylation by mutating serine 6 to alanine. However in this study, mutations were made in the full length wild type SF2 Nef protein. It is known that serine 6

in HIV-1_{SF2} Nef is necessary for the efficient N-terminal membrane myristoylation signal; such that it is hypothesized that mutating serine 6 to alanine will result in a defective protein. We found HIV-1_{SF2} Nef S6A to be functional for PAK-2 activation but the protein levels were significantly destabilized. In order to avoid difficulties in interpretation the HIV-1_{SF2} Nef myristoylation signal was replaced by the myristoylation signal of SIV_{mac239} Nef. HIV-1_{SF2} Nef was mutated at three sites K4A/W5I/K7M to generate HIV-1_{SF2} Nef MGG***AI***SM***RS***, or HIV-1_{SF2} Nef mac-myr. Moreover, changing these amino acids at the N-terminus of HIV-1_{SF2} Nef to the SIV_{mac239} amino acids maintains Nef myristoylation yet eliminates the lysine residues presumed to be necessary for recognition as a protein kinase C site.

HIV-1_{SF2} Nef mac-myr was fully stable and functional with HIV-1_{SF2} Nef mac-myr protein expression being equivalent to HIV-1_{SF2} Nef as shown in the Western blots in Figure 6.4B and Figure 6.5B. Also, the mutant was fully functional for PAK-2 activation and CD4 and MHC-I downregulation.

The findings from these experiments in conjunction with the data presented by the Baur group cast doubt on the functional significance of serine 6 as being a functionally significant PKC site. A recommended future experiment would be to see if the HIV-1_{SF2} Nef mac-myr mutant is phosphorylated in the experimental system used by the Baur group. Specifically, if HIV-1_{SF2} Nef mac-myr were made into a CD8-Nef fusion of only the first 35 amino acids, would it still be

phosphorylated? Based on the data presented in this thesis, it is likely that serine 6 is not a physiologically relevant PKC phosphorylation site. The PKC consensus sequence proposed by the Baur group on HIV-1SF2 Nef as "KWSKRS" is not a strong protein kinase C recognition site (Wolf et al., 2008). Moreover, the arginine at position 8 is highly polymorphic such that arginine is only represent 40% among 1643 subtype B Nef sequences.

The phosphorylation we observed in cells metabolically labeled with ^{32}P may be interpreted as multiple phosphorylation sites on the protein. Thus mutation of one serine will not completely ablate phosphorylation of the whole protein. As shown in Figure 6.3, serine mutagenesis results in removal of individual phosphorylation sites yet the protein was still phosphorylated. One scenario where several serines are phosphorylated is consistent with the global phosphoproteome analyses. Based on studies from the Mann group, it was proposed that protein phosphorylation "is regulated differently on different sites within the same protein… suggesting that protein phosphorylation typically serves different functions of different sites on the protein (Olsen et al., 2006).

It is clear, however, that Nef phosphorylation is a common phenomenon since it has been reported in several HIV-1 Nef alleles as well as SIV Nef. Nef phosphorylation has been demonstrated in many different cell types under many

different experimental conditions. Given such a widespread phosphorylation, it initially appeared that Nef phosphorylation was physiologically relevant.

Is Nef phosphorylation non-functional? An alternative interpretation of these data would be that HIV-1_{SF2} Nef phosphorylation in an example of a non-functional phosphorylation. Nef may fit the criteria of a non-functional phosphorylation as defined by Gustav Lienhard. As suggested by Leinhard, low levels of phosphorylation that are "effectively non-functional" are tolerated because the energy expenditure on the cell for such low levels of phosphorylation are of the order of less than 1% of the cellular ATP pool (Lienhard, 2008). Thus, there is little cellular burden for a functionally null phosphorylation. As a viral protein Nef would be minimally subject to even these lax energetic restrictions.

There is also an evolutionary argument supporting the non-functional phosphorylation of HIV Nef. HIV is an excellent paradigm for studying protein evolution given the high mutability and the necessity for viral replicative fitness. Importantly, only a fraction of replicated virions are viable. As a result of such abortive replication, there is a large pool of non-functional virus. It is estimated that 15% of Nef protein sequences that have been published on NCBI are grossly defective (O'Neill et al., 2006b).

Based on characterization of the human kinome, it is thought that while most human cell types express hundreds of kinases, the cells have not evolved to a high degree of specificity for the a functional substrate. It has been proposed that "cells have not evolved to the point where specificity of each of these kinases is so exquisite that it exclusively phosphorylates substrate proteins" (Lienhard, 2008). Thus it is possible that HIV has not evolved under selective pressure against non-functional Nef phosphorylation. This is supported by the fact that the observed experimental phosphorylations on Nef have occurred on laboratory adapted strains of HIV.

Another argument toward the non-functional phosphorylation of Nef is the lack of conservation of a putative phosphorylation site between HIV-1 and SIV. According to Lienhard, "the lack of conservation of a phosphorylation site in orthologous protein from different species can be considered suggestive evidence that phosphorylation might have no function." Nef is an example of this discrepancy in phosphorylation. Examples of allele specific phosphorylation has been reported (Guy et al., 1987; Laurent et al., 1990). Threonine at position 15 was suggested to be a PKC phosphorylation site. Subsequent mutation of position 15 to alanine resulted in fully functional Nefs. Expression of this T15A mutant Nef in a vaccinia virus system demonstrated the protein was not phosphorylated a

the half-life was dramatically decreased, strongly suggesting that the reported phosphorylation was non-functional (Laurent et al., 1990).

Alternative non-phosphorylation dependent explanation of increased CD4 downregulation in the presence of PMA. Of special note is the ability of PMA to enhance the downregulation of CD4 in a mutant CD4 with serine to alanine mutations in the cytoplasmic tail. The suggested mechanism for this effect was the PMA induced the increased phosphorylation of Nef, and thus the functional consequence was increased CD4 downregulation (Luo et al., 1997a). An alternative explanation is an effect of PMA on trafficking in addition to the accelerated endocytosis of CD4 from the cell surface (Pelchen-Matthews et al., 1992; Pelchen-Matthews et al., 1998). Work from the Marsh group has demonstrated that PMA induced downregulation of CD4 occurs through a complex mechanism with effects in internal trafficking in addition to phosphorylation of the cytoplasmic tail of CD4. In their proposed model, PMA induced CD4 downregulation occurs via increased association of CD4 with clathrin-coated pits. Based on electron microscopy, it was demonstrated that PMA increased CD4 association with clathrin coated pits three-fold (Pelchen-Matthews et al., 1991; Pelchen-Matthews et al., 1993).

In light of these observations, it is possible the observed increase in CD4 downregulation in HeLa.H4 cells upon treatment with PMA is the result of increased clathrin-coated pit association and/or diversion of CD4 to late endosomal sorting compartments. In order to probe this alternative explanation, future recommended experiments would investigate the effect of PMA on Nef regulation of clathrin-mediated endocytosis at the plasma membrane. Previous work from Benichou and Benmerah has used total internal reflection fluorescence microscopy to characterize Nef dynamics at the plasma membrane relative to clathrin (Burtey et al., 2007). However in this study, they did not investigate a potential PMA effect as proposed by the Marsh group. It would be useful to use the imaging experiments described by Benichou and Benmerah to determine how PMA affect Nef mediated CD4 downregulation via clathrin endocytosis and determine whether this is Nef phosphorylation independent.

HIV-1 Nef is not an autophosphorylated GTPase. Despite all the ambiguities of Nef phosphorylation discussed, it is clear however that Nef is not an autophosphorylated GTPase as originally proposed by Guy et al. Based on partial protein sequence homology to v-ras, c-ras, and bovine g-proteins, it was proposed that Nef was a GTP binding protein and was phosphorylated in the presence of γ^{32}P-GTP (Guy et al., 1987). However, this early observation was

discounted by several independent groups. Bacterially expressed recombinant Nef from HIV-1_{BH10} and HIV-1_{LAV1} was used to demonstrate that the GTPase activity of the crude bacterial extracts did not copurify with Nef (Kaminchik et al., 1990). The authors in this study suggested the discrepancy in between their work and Guy et al. could be due to the impurities in the protein preparation or that the GTPase activity observed by Guy et al. was actually contaminants from the insoluble protein fraction. Another possibility is that the two group used different Nef sequences. Another independent group also showed HIV-1_{NL4-3} Nef did not have GTP binding activity using a baculovirus expression system (Matsuura et al., 1991).

Summary and perspectives. Given the multiple phosphorylation states of Nef, it is a distinct possibility that Nef phosphorylation is non-functional. The addition of PMA increases Nef phosphorylation as well as potentiates CD4 downregulation (Luo et al., 1997a). If Nef phosphorylation is non-functional, the increased CD4 downregulation in the presence of PMA may also be phosphorylation independent. Phosphorylation has many diverse effects on cellular function and phosphorylation states of a protein vary greatly depending on cellular stimuli. Nef is an example of such a complex protein, thus complicating elucidation of the mechanisms of phosphorylation regulation.

Chapter 9: Conclusions and Recommendations for the Structural and Functional Analysis of Nef Activation of PAK-2

Characterization of Nef effector domains for PAK-2 activation. HIV-1 Nef structural variants from subtypes B, C, and E all activate PAK-2. Amino acid positions 85, 89, 188 and 191 on HIV-1 Nef have been previously characterized to be involved in the activation of PAK-2 (O'Neill et al., 2006a; O'Neill et al., 2006b). Among 1643 subtype B HIV-1 Nef sequences, the consensus sequence for these four sites is L85, H89, R/K188, and F191 (LHKF). While among 70 subtype E HIV-1 Nef sequences the consensus sequence for these four sites is F85, F89, A188, and R191 (FFAR) (O'Neill et al., 2006b). Among 23 subtype C HIV-1 Nef sequences, the consensus sequence is F85, F89, S188, and R191 (FFSR) while F85, F89, H188, and H191 (FFHH) is another subtype C Nef structural variant (Novitsky et al., 2002; O'Neill et al., 2006a). Altogether, LHKF, FFAR, and FFHH represent three distinct structures of Nef that activate PAK-2. Whether these three structures are interacting with the same host cellular factor of three different factors remains unknown. Furthermore, it is likely that Nef binds an SH3 domain in addition to the targets of LHKF, FFAR, and FFHH. What is known about these two effector domains will be discussed and a tentative model for PAK-2 activation will be proposed.

Other proposed regions of Nef involved in PAK-2 activation. It has been previously proposed that Nef induced activation of PAK-2 can be initiated by at least three different effector domains residing at the same location on the surface of Nef. There is a close 3-dimensional spatial relation between Nef residues 85, 89, 188, and 191. These residues in the HIV-1 subtype B SF2Nef are L85/H89/K188/F191. Only the H89 residue can be considered to be highly conserved at 97% for subtype B Nefs. F191 is moderately conserved at 94% while lysine 188 and leucine 85 are not conserved. The 188 position is mostly arginine (75%) while lysine is only found 4% of the time. With regard to position 85, leucine is the most common residue but is present only 47% of the time. In fact, position 85 is one of the most variable residues in subtype B Nefs (O'Neill et al., 2006b). Not surprisingly mutation of positions 89 or 191 results in Nefs defective for PAK-2 activation, while mutations of position 188 to arginine, histidine, serine, or threonine have small effects on PAK-2 activation (0.5 to 1.5 activity related to SF2Nef).

Characterization of the alternative Nef FFAR structural effector domain. In subtype E Nefs the amino acids found in these four positions are different. 85F, 89F, and 191R appear to be highly conserved. Position 188 can be

alanine or serine. As demonstrated in Figure 7.6, it has been confirmed here that the four amino acids F85, F89, A188 or S188, and R191 support PAK-2 activation on a HIV-1_{SF2} Nef background (O'Neill et al., 2006b). The HIV-1_{SF2} Nef quadruple mutant with F85/F89/A188/R191 (FFAR) has been shown to be shown to be 85% active of HIV-1_{SF2} Nef. The compelling nature of this finding is the restoration of PAK-2-activation defective HIV-1_{SF2} Nef F191R to near full activity by inclusion of the other three amino acids. This has lead to the proposal that these four residues FFAR are representative of a structural domain with PAK-2 activation effector function. By using the shorthand FFAR there is no implication that serine at 188 is less functional or that these are the only four amino acids involved.

Characterization of the alternative Nef FFHH structural effector domain. The original observation that subtype E Nefs has an alternative structural effector domain relative to subtype B Nefs has been extended to FFHH. FFHH is found in a subset of HIV-1 subtype C Nefs. To this end, HIV-1_{SF2} Nef was mutated to F85/F89/H188/H191. The single mutation of HIV-1_{SF2} Nef F191F is devoid of PAK-2 activation while the FFHH exhibits full PAK-2 function. More numerous than FFHH in HIV-1 subtype C Nefs is FFSR (Foster et al., unpublished

observations). Therefore, FFHH is a variant that appears in a relative small number of subtype C Nefs.

While the above observations strongly suggest that there are three separate Nef structures that induce PAK-2 activation, this concept has not been previously confirmed or extended. An opportunity to do both arose with the finding by Agopian et al. that a brain derived allele of Nef, 5C, was hyperactive. This Nef was a rare subtype B Nef with the FFHH signature. The striking feature of HIV-1_{5C} Nef was the mutation of S187I. Agopian et al. demonstrated that mutation of the isoleucine to the prevalent serine (HIV-1_{5C} Nef I187S) resulted in a Nef with similar activity to HIV-1_{SF2} Nef. In other words, the property of hyperactivity was lost.

To further investigate the property of PAK-2 hyperactivity, the S187I mutation was introduced into HIV-1_{SF2} Nef (LHKF), HIV-1_{SF2} Nef FFAR, and HIV-1_{SF2} Nef FFHH. HIV-1_{SF2} Nef S187I lost the ability to activate PAK-2 HIV-1_{SF2} Nef FFAR/S187I was somewhat enhanced in PAK-2 activation, and HIV-1_{SF2} Nef FFHH/S187I was hyperactivated to similar extend as HIV-1_{5C} Nef. These results have two major implications. First, the hyperactivity of HIV-1_{SF2} Nef FFHH/S187I validates the use of the quadruple FFHH mutation in a subtype B background to model HIV-1_{5C} Nef hyperactivity. Second, the different responses

of the three forms of Nef to the S187I mutation support the designation of the three forms as mechanistically distinct.

Further validation of the distinct nature of LHKF, FFAR, and FFHH comes from the observation that HIV-1_{SF2} Nef K188L is also hyperactivated. This unexpected observation was discovered while characterizing the relative common residues found in subtype B Nefs at position 188. Only HIV-1_{SF2} Nef K188L resulted in hyperactivity (Figure 7.2). Insertion of this mutation into FFAR and FFHH gave distinctly different results in that neither of these Nefs was greatly affected.

Future directions for understanding the PAK-2 effector domain on Nef. Ongoing studies of the hyperactivation phenomena will determine the dependence of hyperactivation by S187 in FFHH and L188 in LHKF on hydrophobicity of the substitution. For example, ongoing experiments are investigating the role of a leucine substitution at position 188 in HIV-1_{LAI}, another subtype B Nef. Also it is unknown whether the rarity of the hyperactive form of Nef is a consequence of being deleterious to viral fitness. Future experiments to address this would include assaying HIV-1 single round infectivity of these various Nef structural variants.

It will also be important to determine if the hyperactivation is reflected in PAK-2 trans-kinase activity or if it is exclusively a property of autophosphorylation. Future experiments would include assaying the ability of the different PAK-2 hyperactive Nefs to phosphorylate different cellular substrates such as myelin basic protein. Experiments to characterize differential effects of Nef hyperactivity on PAK-2 substrate phosphorylation of merlin as depicted in Figure 7.8 did not demonstrate mechanistically distinct results. It appears that merlin phosphorylation is not affected by varying levels of PAK-2 activation levels by Nef. Important to note is that merlin is phosphorylated by PAK-2 on only one site (serine 518), however other PAK-2 substrates such as myelin basic protein are phosphorylated by PAK-2 on many sites. Thus it will be interesting to compare a singly phosphorylated cellular substrate to a multiply phosphorylated substrate.

What is the functional significance of leucine at position 188? Another important observation from this study was that 31 Nef sequences out of 1643 HIV-1 subtype B sequences (O'Neill et al., 2006b) that contained leucine at position 188. Given that the substitution of leucine at position 188 results in PAK-2 hyperactivity (Figures 7.6 and 7.7), the next salient question to ask whether this hyperactivity has a consequence on disease progression. These 31 Nef sequences

were obtained from various different patients (Michael et al., 1995; Wang et al., 2000) with different and divergent rates of disease progression. Some of these leucine 188 patient sequences came from long-term non progressors while other came from intermediate progressors. Thus future experiments will involve expressing these full length patient isolate Nef isolates and to assay PAK-2 activation levels in order to correlate PAK-2 hyperactivity to disease progression.

Further analysis of these 31 sequences with leucine at position 188 showed that these sequences appeared to be close to the overall consensus sequence for subtype B Nefs except for position 85 which was skewed in favor of arginine, as depicted in Figure 9.1. Using this compilation of the 31 leucine 188 sequences, subsequent experiments can be performed to further investigate the amino acid cooperativity between the different sites in the context of PAK-2 hyperactivation.

1. 31M
2. 31G
3. 31G
4. 30K, 1R
5. 31W
6. 31S
7. 31K
8. 12R, 10G, 6S, 2C, 1-
9. 20S, 11N
10. 15V, 11L, 2-, 1G, 1I, 1M
11. 12G, 8V, 6F, 5I
12. 31G
13. 31W
14. 30P, 1A
15. 13A, 11K, 6T, 1I
16. 29V, 2I
17. 31R
18. 31E
19. 30R, 1S
20. 31M
21. 13R, 10K, 7E, 1T
22. 17R, 11K, 2Q, 1-
23. 29A, 1I, 1T
24. 31E
25. 31P
26. 29A, 2P
27. 31A
28. 22E, 5K, 3D, 1A
29. 31G
30. 31V
31. 31G
32. 31A
33. 20V, 8A, 3P
34. 31S
35. 30R, 1L
36. 31D
37. 31L
38. 23E, 7A, 1D
39. 30K, 1Q
40. 30H, 1R
41. 31G
42. 31A
43. 20I, 11L
44. 31T
45. 22S, 8N, 1G
46. 31S
47. 31N
48. 26T, 5-
49. 25A, 5-, 1T
50. 28A, 3T
51. 21N, 10T
52. 31N
53. 31A
54. 19A, 12D
55. 31C
56. 31A
57. 31W
58. 31L
59. 30E, 1Q
60. 30A, 1V
61. 31Q
62. 30E, 1K
63. 13D, 9E, 9N
64. 30E, 1G
65. 22E, 9D
66. 30V, 1G
67. 31G
68. 31F
69. 31P
70. 31V
71. 21R, 9K, 1F
72. 31P
73. 31Q
74. 29V, 2E
75. 31P
76. 28L, 3V
77. 31R
78. 31P
79. 31M
80. 30T, 1S
81. 28Y, 2R, 1F
82. 16R, 15K
83. 29A, 2G
84. 31A
85. 16R, 11V, 2L, 2M
86. 30D, 1Y
87. 29L, 2F
88. 30S, 1T
89. 31H
90. 30F, 1L
91. 31L
92. 30K, 1R
93. 31E
94. 19K, 12E
95. 31G
96. 31G
97. 31L
98. 27E, 4D
99. 31G
100. 31L
101. 31I
102. 16Y, 14H, 1W
103. 31S
104. 31Q
105. 12K, 11Q, 8R
106. 31R
107. 31Q
108. 30D, 1E
109. 31I
110. 31L
111. 31D
112. 31L
113. 31W
114. 17V, 14I
115. 31Y
116. 31H
117. 31T
118. 27Q, 4E
119. 31G
120. 30Y, 1F
121. 31F
122. 31P
123. 31D
124. 29W, 2G
125. 30Q, 1H
126. 28N, 2K, 1S
127. 31Y
128. 31T
129. 30P, 1K
130. 31G
131. 31P
132. 31G
133. 13V, 11I, 7T
134. 29R, 1G, 1T
135. 28Y, 3F
136. 31P
137. 31L
138. 27T, 3C, 1A
139. 27F, 4Y
140. 28G, 3E
141. 30W, 1L
142. 31C
143. 30F, 1L
144. 30K, 1E
145. 31L
146. 28V, 2L, 1M
147. 31P
148. 31V
149. 29E, 2D
150. 22P, 9K
151. 30E, 1G
152. 13Q, 12E, 6K
153. 30V, 1I
154. 30E, 1K
155. 17E, 14K
156. 31A
157. 31N
158. 20E, 10K, 1G
159. 31G
160. 31E
161. 29N, 1D, 1K
162. 31N
163. 20S, 11C
164. 31L
165. 31L
166. 31H
167. 31P
168. 30M, 1I
169. 31S
170. 18L, 13Q
171. 31H
172. 31G
173. 21M, 10V
174. 15D, 15E, 1G
175. 31D
176. 18P, 12T, 1S
177. 31E
178. 16K, 12G, 2R, 1E
179. 31E
180. 31V
181. 31L
182. 15A, 9M, 4V, 2K, 1E
183. 31W
184. 26K, 5R
185. 31F
186. 31D
187. 29S, 2G
188. 31L
189. 30L, 1P
190. 31A
191. 31F
192. 12R, 11N, 5H, 2P, 1Q
193. 29H, 2P
194. 19V, 12M
195. 30A, 1G
196. 31R
197. 31E
198. 23L, 8K
199. 31H
200. 31P
201. 31E
202. 31Y
203. 31Y
204. 31K
205. 29D, 2N
206. 31C

Figure 9.1. 31 subtype B HIV-1 Nef sequences containing leucine at position 188. Compilation of 31 subtype Nef sequences containing leucine at position 188 in red. The amino acid positions 1 through 206 of the Nef protein are shown. For each position, the number of different sequences containing the indicated amino acid residue is shown followed by its letter designation.

Previous characterization of SH3 binding to Nef. Saksela et al. first characterized SH3 binding to HIV-1 and SIV Nef via the highly conserved PxxP polyproline motif. This was demonstrated upon Nef binding to the SH3 domain of Hck from U937 monocytes (Saksela et al., 1995). Important to note is that Hck is only endogenously expressed in monocytes and macrophages but not in T cells while Nef is expressed in monocytes, macrophages, and T cells. Saksela later demonstrated by surface plasmon resonance that HIV-1 Nef bound to Hck SH3 at a high affinity of 250 nM. This highly affinity binding only occurred with full length HIV-1 Nef (Lee et al., 1995).

The three dimensional structure of the structural core of Nef by X-ray crystallography (Lee et al., 1996). The core consists of roughly amino acids 71-148 and 178-203. The first 70 amino acids of Nef, the internal loop from 149-177, and the last three amino acids are not part of Nef's globular structure. The internal loop of 29 amino acids may be essentially random coil. From the crystal structure of the Nef core, it was further confirmed that Nef interacts with an SH3 domain using a polyproline type II helix (Lee et al., 1996). The core domain of Nef (71-148 and 178-203) was co-crystallized with the SH3 domain of FynR96I. The reason that a mutated SH3 domain from Fyn was used was technical but reflects interesting properties of the RT loops of the Hck and Fyn Sh3 domains. At position 96 Hck is isoleucine but Fyn is arginine. Hck binds tightly to Nef but Fyn

does not (Lee et al., 1996). Replacing this arginine in Fyn resulted in an SH3 domain with high affinity for Nef as well as a binding partner for Nef core that gave analyzable crystals (Lee et al., 1996). Therefore, the binding of Fyn SH3 domain with R96I mutation is used as a model for Nef ability to interact with Hck and possibly other SH3 domains.

It has also been postulated that myristoylated Nef forms a compact structure with the N-terminal segment of packed against the surface of the core with the myristate group bound to a hydrophobic groove on the surface (Geyer et al., 1999). This proposal is supported by the finding that HA or myc tags inserted between amino acids 24 and 25 cannot be immunoprecipitated by a corresponding monoclonal (Raney et al., 2007). While the N-terminal segment and internal loop are required for CD4 downregulation they are not required for the binding of Nef to the SH3 domain of Hck (Lee et al., 1995). The binding of intact Nef to the Hck SH3 domain is high affinity (250 nM), but the significance of this interaction is unclear since Hck is not endogenously expressed in T cells (Lee et al., 1995).

Lee et al. described the three dimensional core of Nef as consisting of three layers. The "top" layer consists of a polyproline helix consisting of amino acids 71-77, followed by two alpha helices of 14 and 14 amino acids (Lee et al., 1995). Following the canonical SH3 binding segment ($_{72}$PQVPLR$_{77}$) is proline 78 that kinks the connecting sequence between the polyproline helix and the first alpha

helix, such that the helices run in roughly anti-parallel to each other. The loop between the first and second helices bends the second helix back in the direction of the polyproline helix, such that the C-terminal half of the helix interacts with polyproline helix. This creates a pocket between the three helices that is lined with exposed hydrophobic residues. The second layer consists of four anti-parallel beta-sheet like strands of irregular conformation. Packed under this layer are two short alpha helices. Only the N-terminal layer interacts with FynR96I. The SH3 binding surface is a relatively flat beta sheet barrel with hydrophobic residues aligned to bind to a polyproline helix. Of crucial importance is the RT loop that extends out of the barrel to interact with the hydrophobic pocket formed by the three helices just C-terminal of the Nef polyproline helix (Lee et al., 1995).

The role of SH3 binding in Nef activation of PAK-2 has been previously investigated by mutating PxxP to AxxA. From these structure based mutational analyses, it was proposed that SH3 binding to Nef is required for PAK-2 activation (Manninen et al., 1998) In addition to proline 72, proline 75 and arginine 77 other regions of Nef were next investigated to further elucidate the role of SH3 binding on Nef function. Work from the Guatelli group mutated residues F90, W113 and I114 to alanine. These three residues constitute a hydrophobic pocket in Nef thought to be where the hydrophobic RT-loop of the Hck SH3 is buried (Craig et al., 1999; Lee et al., 1995). Alanine mutagenesis of these putative SH3 binding

residues caused “subtle impairments on protein expression” which prevented the authors from determining the functional significance of these sites.

Phage display analysis of the complete human SH3 proteome was performed to look for binding to HIV-1 Nef and PAK-2. The most important finding of this study was that the only SH3 binding to Nef observed was through Hck. This is rather puzzling given that Hck is only expressed in monocytes and macrophages, moreover “T cells do not encode a postulated Hck-like high-affinity SH3 ligand for Nef” (Karkkainen et al., 2006). Instead, the authors proposed that “the role of SH3-directed protein complexes of Nef in T cells may be fundamentally different and not require such tight binding.” Although the 250 nM high-affinity binding of Hck SH3 to Nef is observed in vitro in macrophages, in T cells the possibility of tight binding to an SH3 domain remains unproven and whether PAK-2 activation requires a host cellular SH3 domain protein remains unknown.

Prior characterization of Nef polyproline domain contribution to MHC-I downregulation. This study is particularly pertinent given recent papers that have investigated the contribution SH3 binding to Nef for MHC-I downregulation. It was previously thought that an intact polyproline motif on Nef is necessary for MHC-I downregulation (Greenberg et al., 1998). Unfortunately,

the error in this study was mechanistic interpretation of the mutation of $_{72}$PQVPLR$_{77}$ to $_{72}$***A***QV***A***LR$_{77}$. This drastic mutation disrupted the local secondary structure to cause the functional defect. It is known that mutations in the Nef polyproline motif will have deleterious effects on multiple functions of Nef, but in the case of MHC-I downregulation by Nef, it was demonstrated that proline 78 was the critical residue. Instead, single mutation of to $_{72}$***A***QVPLR$_{77}$ and to $_{72}$AQV***A***LR$_{77}$ only had minimal effects on MHC-I downregulation, demonstrating proline 72 and proline 75 were not as significant (Yamada et al., 2003). More recent work demonstrated that proline 78 of Nef regulates MHC-I downregulation by an SH3 independent mechanism (Casartelli et al., 2006).

Conservative mutations in the HIV-1$_{SF2}$ Nef polyproline helix. In light of these previous structural studies of Nef binding to SH3 domains, a fine structure mutational analysis was employed in this thesis. Unlike previous studies using alanine mutagenesis, this study employed conservative mutation of the $_{72}$PQVPLR$_{77}$ residues. As demonstrated in Figure 7.14, HIV-1$_{SF2}$ Nef mutations P72G, Q73R, P75G, L76V, and R77K have deleterious effects on PAK-2 activation while protein expression is maintained. The HIV-1$_{SF2}$ Nef V74I mutation actually enhanced PAK-2 activation. This is in contrast to previous studies where mutations of P72A, P75A, and R77A resulted “detrimental effects

on protein expression" (Craig et al., 1999). In this study, the mutation of proline to glycine and arginine to lysine maintained protein expression as shown in the Western blot in Figure 7.14B. Based on the BLOSUM 62 substitution matrix (Henikoff and Henikoff, 1992), these amino acid substitutions, except for proline to glycine, are more well tolerated evolutionarily than the alanine substitutions. Moreover, these conservative mutations allowed for functional understanding of these contributions of these residues on Nef to PAK-2 activation. As shown in the autoradiography in Figure 7.14A, P72G, Q73R, P75G, L76V, and R77K disrupted PAK-2 activation. Given the previously discussed model of Nef binding to an SH3 domain, these data suggest intact SH3 binding is necessary for PAK-2 activation.

Proposed model of HIV-1 Nef binding to SH3. Based on the crystal structure of the conserved core of Nef complexed to SH3, the Nef polyproline helix binds the FynR96I SH3 domain in the minus orientation with valine 74 being designated as P_0 (Lee et al., 1996). Based on this description of the Nef/SH3 binding domain, it is proposed that the same structure is required for PAK-2 activation. In this model valine 74 interacts with hydrophobic residues in the SH3 domain. Interestingly, increasing the hydrophobicity of the residue by substituting isoleucine increases the PAK-2 activation of Nef (Figure 7.14). The proline

adjacent to valine (P_{-1}) and the proline on the other side of valine (P_2) also engage the SH3 domain with hydrophobic interactions. Mutation of either of these prolines to alanine prevents Nef binding to the FynR96I SH3 domain (Lee et al., 1996). Mutation of these residues to glycine virtually eliminates the ability of Nef to activate PAK-2. These results are consistent with an SH3 domain protein being part of the Nef/PAK-2 activation complex.

It is also interesting that mutations Q73R and R77K (P_1 and P_{-3}, respectively) also effectively eliminate the PAK-2 activation function (Figure 7.14). Arginine 77 has a complex role in the binding of Nef polyproline helix to SH3. The side chain of arginine 77 stacks closely with the face of a tryptophan residue in the SH3 binding surface and extends to allow the formation of a salt bridge with an aspartate within the SH3 domain but outside of the hydrophobic face. That the conservative mutation of arginine 77 to lysine eliminates PAK-2 activation is most simply rationalized as the disruption of Nef binding to an SH3 domain protein required for PAK-2 activation.

Glutamine 73 also has important side chain interactions as it binds to the backbone of the first alpha helix. The strong positive charge of arginine and its larger size that glutamine could very well disrupt the structure of the Nef protein in this region. Based on the crystal structure of the conserved core of Nef complexed to SH3, the authors suggest that several internal interactions between

the polyproline and the backbone of the first alpha helix stabilize the position of arginine 77 (Lee et al., 1996).

A final observation that is consistent with a Nef SH3 domain interaction is the relatively weak impact of mutating leucine 76 to valine. In the polyproline helix, the leucine 76 side chain points away from the hydrophobic surface of the SH3 domain. The backbone amide of leucine 76 does interact with a carbonyl group in the backbone of the C-terminal end of the second alpha helix. Therefore, this study has analyzed all six residues of the polyproline of Nef and found no clear exception to the conclusion that Nef activation of PAK-2 requires an SH3 binding partner for the activation of PAK-2 (Figure 7.14). There is still the question of whether or not a hydrophobic residue in the RT loop of the unknown SH3 binding protein is required to increase the affinity of the interaction. Future studies will be necessary to explore the role of the hydrophobic pocket of Nef formed between the polyproline and two alpha helices. This pocket is clearly necessary for Hck binding to Nef but the role of these Nef residues is not yet known for PAK-2 activation.

Does Nef activation of PAK-2 require a weak or strong SH3 binding?

Based on the data shown in this thesis, it is clear that an intact polyproline helix is necessary for PAK-2 activation, thus suggesting SH3 binding is necessary for Nef

activation of PAK-2. These data support the model proposed by Saksela that the SH3 binding involved in Nef activation of PAK-2 is a weak affinity interaction. All of the experiments shown in the thesis have been conducted with either T cells or HEK 293T cell, none of which express Hck. Thus Hck SH3 binding is not involved in Nef activation of PAK-2. There must be another SH3 containing protein involved in the Nef/PAK-2 complex. The SH3 domain of this unknown protein must binding Nef weakly since no other protein was identified by Saksela et al. in their phage display experiments (Karkkainen et al., 2006). A weak but necessary SH3 binding supports a model where Nef activation of PAK-2 occurs via a ternary complex of Nef/PAK-2 and an unidentified SH3 protein.

Proposed model for Nef activation of PAK-2: a ternary complex of SH3/Nef/PAK-2 binding. The work presented in this thesis has characterized a PAK-2 effector domain on Nef constituted by amino acid position 85, 89, 187, 188 and 191, illustrated in Figure 7.1. Changes in amino acid composition on the effector domain of Nef caused drastic changes in PAK-2 activation levels as quantitated in Figure 7.7. However this is not the only region on Nef that regulates PAK-2 activation. The highly conserved polyproline helix highlighted the yellow and green in Figure 7.11 also plays a regulatory role in the activation of PAK-2. Mutations of this SH3 binding region completely abrogate PAK-2

activation (Figure 7.14) suggestion intact SH3 binding is necessary, albeit a weak binding. Altogether, these data suggest a speculative model where activation of PAK-2 by Nef requires a ternary, or higher, complex of SH3/Nef/PAK-2 binding.

First, Nef may bind weakly to an unidentified SH3 containing protein. The binding of the SH3 protein must be stabilized for subsequent interaction with PAK-2. The required stabilization may be provided by interaction of the unknown SH3 domain protein with LHKF, FFAR, or FFHH. Alternatively, it is also possible that the required stabilization is provided by a fourth unknown protein. Also, the difference in the nature of the stabilization between the three structural variants is unknown. In other words, it is possible that the stabilizing protein for LHKF, FFAR, and FFHH may bind each via alternative orientations or the same protein. Upon stabilization, this ternary complex can activate PAK-2.

This proposed model is supported by previous work suggesting the SH3 binding to Nef allosterically regulates Nef. As proposed by Guatelli and Saksela., the hydrophobic RT-loop of the Hck SH3 domain is buried in the hydrophobic pocket on Nef constituted by F90, W113, and I114 thus stabilizing the SH3/Nef interaction (Craig et al., 1999). As shown in Figure 7.14, mutations in this interaction are thought to allosterically disrupt the PAK-2 effector domain on Nef. Also, it is noteworthy that isoleucine 114 is part of the SH3 domain, thus it was present in the phage display library screen performed by Saksela (Karkkainen et

al., 2006). In the model proposed in this thesis, the stabilization requires an extra SH3 domain binding site and would thus explain why it was not identified in the phage display screen.

Precedent for Nef ternary regulatory binding: the Nef/MHC-I/AP-1 complex. This proposed ternary complex of SH3/Nef/PAK-2 binding for PAK-2 activation is supported by work from the Collins group. MHC-I downregulation by Nef is thought to involve a ternary complex of Nef, MHC-I, and AP-1, as shown in Figure 2.1. Nef binding the cytoplasmic tail of MHC-I is mediated through the Nef acidic domain ($_{62}$EEEE$_{65}$), the polyproline helix ($_{72}$PQVPLR$_{77}$), and methionine 20. This binding then requires Y320 on the cytoplasmic tail of MHC-I to form the Nef binding pocket and sequentially allows for MHC-I binding to AP-1. Importantly, the first step of this ternary binding is mediated through the polyproline motif. Similarly, it is proposed that Nef binding to SH3 via the polyproline helix ($_{72}$PQVPLR$_{77}$) allows for the sequential binding of PAK-2 to Nef via the hydrophobic effector domain constituted by position 85, 89, 187, 188 and 191.

Summary and perspectives. Altogether, this study has provided greater insight into the structural determinants of Nef activation of PAK-2. Work in the

HIV field over the past 10 years has clearly demonstrated that Nef activation of PAK-2 is an important cellular activity of Nef. Unfortunately, it is still poorly understood what the exact structural requirements for PAK-2 activation. O'Neill et al. identified a PAK-2 effector domain on Nef constituted by amino acid positions 85, 89, 188 and 191. This study has extended these initial findings to further characterize this PAK-2 effector domain on Nef and the contribution of position 187 to PAK-2 hyperactivity. Moreover, this study has investigated the necessity of an intact SH3 binding domain on Nef for PAK-2 activation. Based on the work of these studies, this thesis proposes a model whereby Nef activation of PAK-2 is regulated by a ternary complex of Nef binding weakly to an SH3 domain protein and Nef binding to PAK-2. Future directions will further dissect the determinants of this complex, specifically the importance of hydrophobicity to these interactions.

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