The EMBO Journal Vol.16 No.4 pp.673–684, 1997

Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling

A.John Iafrate[1], Scott Bronson and Jacek Skowronski[2]

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and [1]Department in Molecular Genetics and Microbiology, SUNY at Stony Brook, Stony Brook, NY 11794, USA

[2]Corresponding author

The Nef protein alters T cell receptor (TCR) signaling in T cells and is critical for the pathogenesis of AIDS. We used a transient expression assay in a human CD4$^+$ T cell line to analyze the interaction of Nef with the TCR machinery. We show that, in addition to down-regulating CD4 expression on the cell surface, Nef blocks a receptor-proximal event in CD3 signaling. Analysis of a large number of mutant Nef proteins demonstrated that the effects of Nef on CD4 expression and on CD3 signaling are separable. The ability of Nef to block CD3 signaling was selectively abolished by mutations in the central part of the Nef protein and in particular by those known to disrupt the SH3 binding surface in the structured core of Nef. In contrast, the ability of Nef to down-regulate CD4 expression was selectively abolished by two clusters of mutations, one in the N-terminal and one in the C-terminal region of Nef. These two regions correspond to the two flexible loops in Nef as predicted by solution NMR analysis. We show that this general functional organization is conserved between the Nef proteins of the human and simian immunodeficiency viruses (HIV-1 and SIV). Our data demonstrate that Nef has at least two independent mechanisms to alter TCR function and thus may interfere with a range of T cell responses.

Keywords: CD4/Nef/T cell activation/T cell receptor

Introduction

Evidence indicates that the *nef* gene of simian and human immunodeficiency viruses (SIV and HIV-1) is critical for AIDS pathogenesis. Experiments in SIV-infected rhesus monkeys show that there is a strong selective pressure on the virus to maintain a functional *nef* open reading frame. Animals infected with SIV viruses carrying large deletions in *nef* exhibit low viral loads and do not develop AIDS (Kestler *et al.*, 1991). Moreover, studies of long-term survivors of HIV-1 infection also indicate that *nef* can be an important determinant of clinical outcome. In several long-term non-progressors, the efficient control of HIV-1 infection is associated with deletions in *nef* (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995) or with an unusually high frequency of defective *nef* alleles and functionally defective Nef proteins (Mariani *et al.*, 1996).

The critical function of Nef *in vivo* is not known. *In vitro* Nef enhances viral replication in peripheral blood mononuclear cells and alters signal transduction when expressed in several cell types (de Ronde *et al.*, 1992; De and Marsh, 1994; Miller *et al.*, 1994; Spina *et al.*, 1994). Importantly, Nef can perturb signal transduction in T cells (Garcia and Miller, 1991; Luria *et al.*, 1991; Skowronski *et al.*, 1993; Baur *et al.*, 1994; Du *et al.*, 1995; Graziani *et al.*, 1996). In transgenic mice, Nef expression in T cells blocks the development of CD4$^+$ T cells (Brady *et al.*, 1993; Skowronski *et al.*, 1993) and the infrequent mature CD4$^+$ T cells found in the peripheral lymphoid tissues show elevated expression of activation markers (Lindemann *et al.*, 1994). Moreover, transgenic thymocytes expressing Nef respond with elevated mitogenic and calcium responses to T cell receptor (TCR) stimulation with an anti-CD3 monoclonal antibody (MAb) (Skowronski *et al.*, 1993). In contrast, in human T cell lines, constitutive Nef expression is associated with reduced IL-2 gene expression in response to anti-CD3 MAb (Luria *et al.*, 1991; Collette *et al.*, 1995). Interestingly, in human Jurkat T cells, a CD8–Nef fusion protein can suppress or promote the activation of T cells depending on its intracellular localization (Baur *et al.*, 1994). The opposing consequences of Nef expression in T cell lines *in vitro* and in primary mouse CD4$^+$ T lymphocytes *in vivo* may reflect differences in the nature of the Nef–TCR interaction between these cells.

How Nef alters TCR/CD3 signaling is not known, but genetic and biochemical studies have begun to define possibly relevant interactions between Nef and cellular proteins. One such interaction is between Nef and the CD4 co-receptor (Garcia and Miller, 1991; Aiken *et al.*, 1994; Salghetti *et al.*, 1995). The extracellular domain of CD4 binds the major histocompatibility complex class II molecule and the cytoplasmic domain of CD4 binds the Lck protein tyrosine kinase; these two interactions enhance antigen-specific TCR signaling by several orders of magnitude (Janeway, 1992; Weiss and Littman, 1994). Nef targets these two interactions of CD4 both by inducing the endocytosis and degradation of CD4 in lysosomes and by disrupting the association of CD4 with Lck (Aiken *et al.*, 1994; Anderson *et al.*, 1994; Rhee and Marsh, 1994; Salghetti *et al.*, 1995). The effect of Nef on CD4 and on the CD4–Lck complex was observed with natural SIV and HIV-1 Nef proteins and therefore reflects a conserved function of the viral protein (Anderson *et al.*, 1993; Benson *et al.*, 1993; Mariani and Skowronski, 1993).

Two biochemical interactions of Nef have been described that putatively link Nef to signal transduction pathways. One is with the SH3 domains of the Hck and Lyn protein tyrosine kinases of the Src family and involves the conserved PxxP motif in Nef (Saksela *et al.*, 1995). The other interaction is with the p62 serine kinase of the

p21-activated kinase (PAK) family (Sawai *et al.*, 1994, 1995; Nunn and Marsh, 1996). The Nef–PAK association was implicated as important for the effect of Nef on signal transduction in T cells (Baur *et al.*, 1994). Both of these interactions have mapped to the highly conserved core of the Nef molecule. The crystal and NMR structures of the Nef core that interacts with SH3 domains were solved recently (Grzesiek *et al.*, 1996; Lee *et al.*, 1996).

Since TCR signals that follow from antigen recognition events are involved in the regulation of all of the major aspects of T lymphocyte function, the interaction of Nef with the TCR machinery is likely to be important for AIDS pathogenesis. Here we describe the molecular determinants of the effect of Nef on two aspects of the TCR machinery; CD4 surface expression and CD3 signaling. Using a transient expression assay to characterize a large panel of mutant HIV-1 and SIV Nef proteins, we found that: (i) Nef blocks a receptor-proximal event in the CD3 pathway; (ii) the effects of Nef on CD4 surface expression and on CD3 signaling are independent; (iii) distinct regions of the Nef molecule are important for the effect on CD4 expression and on CD3 signaling; (iv) association with the p62 kinase does not correlate with the effects of Nef on CD4 down-regulation or on CD3 signaling. Our studies define novel molecular interactions of the Nef molecule that are important for the effects of Nef on the TCR machinery and suggest a structure–function model for the Nef protein.

Results

Experimental design: transient assay of the effect of Nef on the TCR machinery

The effects of Nef on CD4 surface expression and on TCR-mediated signaling were studied using a transient expression assay in the JJK $CD4^+$ subline of Jurkat T cells. The JJK cells were transfected with HIV-1 Nef or SIV Nef expression plasmids and then stimulated with the mitogenic anti-CD3 MAb HIT3A. Since stimulation of T cells via TCR/CD3 results in rapid induction of very early activation antigen (CD69) expression on the cell surface (Yokoyama *et al.*, 1988; Ho *et al.*, 1994), induction of CD69 surface expression provided a convenient indicator of CD3 signaling. Flow cytometric analysis of CD4 and CD69 expression permitted quantitation of the effect of Nef on CD4 and CD3 signaling simultaneously on a cell by cell basis.

As shown in Figure 1, transient transfection of the previously characterized natural HIV-1 *nef* allele NA7 (Mariani and Skowronski, 1993) resulted in an ~20- to 50-fold decrease in CD4 expression on the surface of positively transfected Nef-expressing cells ($CD4^{low}$ cells; compare panels 1 and 2). A similar decrease in CD4 expression was observed following transfection with the mac239 *nef* allele from a pathogenic SIV strain (Regier and Desrosiers, 1990) (compare panels 1 and 3). These observations are in agreement with the previously reported ability of both alleles to down-regulate surface expression of CD4 (Benson *et al.*, 1993; Mariani and Skowronski, 1993).

Exposure of JJK cells to anti-CD3 MAb resulted in a readily detectable 3- to 7-fold increase in CD69 surface expression (compare panels 1 and 4). A similar increase

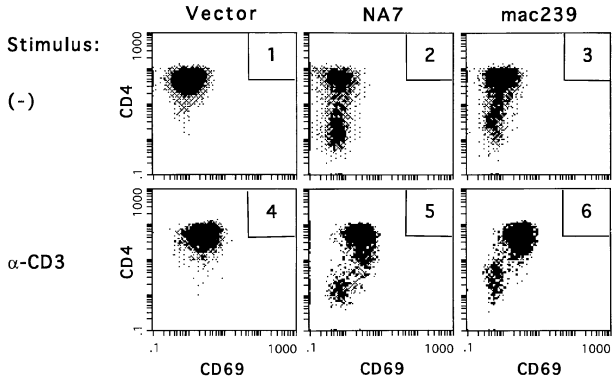


Fig. 1. Transient assay of the effect of Nef on the TCR machinery: two color analysis of CD4 and CD69 antigen expression. Cells were transfected with 20 μg plasmids expressing the NA7 HIV-1 (panels 2 and 5) or the mac239 SIV (panels 3 and 6) *nef* alleles or a control empty vector (panels 1 and 4) and cultured overnight in the absence (–) or presence (α-CD3, 25 ng/ml) of HIT-3A anti-CD3 MAb.

in CD69 expression was observed in untransfected subpopulations ($CD4^{high}$) from cultures electroporated with NA7 and mac239 Nef expression vectors (compare panel 2 with 5 and panel 3 with 6 respectively). In contrast, the positively transfected Nef-expressing $CD4^{low}$ cells from the same cultures did not respond with increased CD69 expression. Thus, both HIV-1 and SIV Nef can block CD3-initiated signaling events that are required for the induction of CD69 expression in Jurkat T cells.

Nef blocks an early event in CD3 signaling

Experiments were performed to localize within known signal transduction pathways the Nef-mediated block to induction of CD69 expression by anti-CD3 MAb. Stimulation of T cells through the TCR results in activation of intracellular signal transduction mechanisms, including activation of both protein kinase C (PKC) and calcium-dependent responses. Since CD69 expression can be triggered by treating cells with PKC agonists (PMA) and calcium ionophores (A23187), we assayed the effect of Nef on CD69 induction by these two agents. As shown in Figure 2A, exposure of Jurkat T cells to PMA or A23187 resulted in CD69 induction similar to that induced by anti-CD3 MAb (compare panel 1 with panels 2–4). In marked contrast to anti-CD3 MAb-stimulated cells, CD69 expression levels were comparable between the Nef-expressing $CD4^{low}$ cells and the non-transfected $CD4^{high}$ population, following PMA or A23187 stimulation (see panels 7 and 8). Control experiments demonstrated that $CD4^{low}$ cells selectively expressed a co-transfected CD20 marker and therefore represented the positively transfected cell population (see panels 9–12). Though it is possible that PMA- and ionophore-induced CD69 expression may involve parallel (CD3-independent) pathways, our observations suggest that Nef may block events upstream of PKC activation and upstream of cellular mechanisms regulated by an increase in intracellular calcium.

Since TCR signaling involves the activation of several downstream effector molecules, including Lck, Rac, Raf and Ras (Weiss and Littman, 1994; Zenner *et al.*, 1995), we asked which of these four molecules can induce CD69 expression. As shown in Figure 2B, transient expression of activated forms of Rac, Raf and Ras in Jurkat T cells

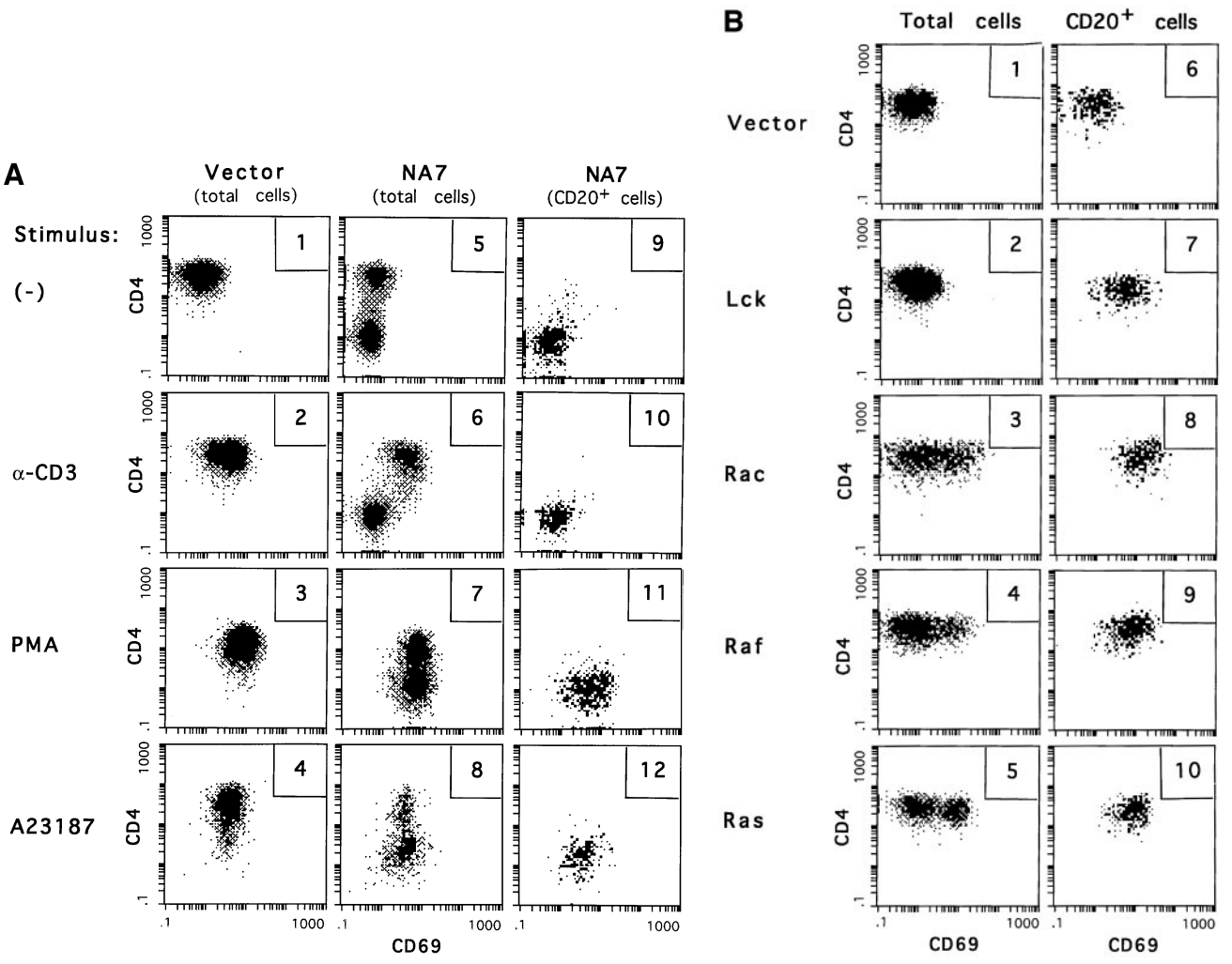


Fig. 2. Nef blocks a receptor-proximal event in CD3 signaling. (**A**) Nef does not block the induction of CD69 expression by phorbol ester or by a calcium ionophore. Flow cytometric analysis of CD4 and CD69 expression in cells transfected either with 20 μg plasmids expressing NA7 Nef (NA7, panels 5–12) or with a control empty vector (Vector, panels 1–4) and 3 μg vector expressing the CD20 marker. Cells were then cultured overnight in medium containing anti-CD3 MAb (α-CD3, 25 ng/ml), phorbol myristate acetate (PMA, 5 ng/ml), calcium ionophore A23187 (A23187, 0.2 μg/ml) or in medium alone (–). CD4 and CD69 expression on all cells that survived electroporation [(total cells), panels 1–8] or on CD20-positive cells [(CD20$^+$ cells), panels 9–12] is shown. (**B**) Activated Lck, Rac, Raf and Ras induce CD69 antigen expression. Jurkat T cells were co-transfected with 5 μg plasmid expressing activated forms of mouse Lck (panels 2 and 7), Rac (panels 3 and 8), Raf (panels 4 and 9), Ras (panels 5 and 10) or a control empty vector (panels 1 and 6) and 3 μg CD20 reporter plasmid. CD4 and CD69 expression on cells surviving electroporation is shown on the left (Total cells) and that on CD20-positive cells is shown on the right (CD20$^+$ cells).

all resulted in efficient induction of CD69 in positively transfected cells (compare panels 3–5 with 1 and 8–10 with 6). The activated form of Lck was also effective, although fewer cells showed a comparable increase in CD69 expression (Figure 2B, see panels 2 and 7). The indiscriminate induction of CD69 antigen expression by a wide variety of biochemical effectors and pharmacological agents suggests that the CD69 gene is a target for several different pathways that branch downstream of TCR. Thus, efficient blocking by Nef of such a promiscuously responsive gene as CD69 indicates that Nef disrupts an early event in CD3 signaling, possibly by interfering with the recruitment of signaling molecules to the receptor.

The effects of Nef on CD4 surface expression and on CD3 signaling are independent

To assess which amino acid residues in Nef contribute to its effects on CD4 expression and on CD3 signaling and to determine whether these effects reflect separate functions of the Nef protein, we analyzed a panel of both novel and previously studied mutant HIV-1 and SIV Nef proteins (Mariani and Skowronski, 1993; Saksela *et al.*, 1995; Aiken *et al.*, 1996; Mariani *et al.*, 1996). Thirty six mutations located in the highly conserved central region and in the less conserved N-terminal and C-terminal regions of Nef were studied (see Figure 3 for the definition of the mutagenized regions of Nef). These experiments defined four functional classes of mutations in Nef, the primary data for representatives of which are shown in Figure 4 (complete data for all mutants are shown in Figures 5–7). The first class of mutations, represented by a lysine substitution for E174, disrupted the ability of NA7 Nef to down-regulate CD4 expression, but did not have a detectable effect on the ability of Nef to block CD3 signaling (Figure 4, compare panel 3 with 1 and 2 and panel 8 with 6 and 7). The second class, typified by alanine substitutions for both the P72 and the P75 residues, preferentially disrupted the ability of Nef to block CD3

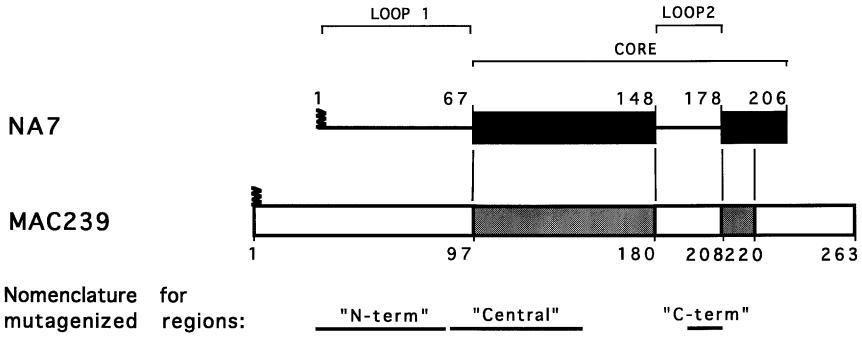


Fig. 3. Schematic representation of HIV and SIV Nef proteins. The disordered regions of HIV-1 Nef spanning amino acids 1–67 and 149–178 are represented by a line and designated Loop 1 and Loop 2 respectively. The structured regions of the HIV-1 Nef core spanning residues 68–148 and 179–206 are represented by solid bars (derived from NMR and crystal structures; Grzesiek *et al.*, 1996; Lee *et al.*, 1996). N-terminal myristoylation is also shown ( ). Conserved structural elements of the SIV mac239 Nef protein that show at least 65% amino acid identity with NA7 Nef are represented by gray bars (amino acids 98–180 and 209–220). The remaining mac239 Nef sequences, spanning residues 1–97, 181–208 and 221–263 show <15% amino acid identity with the homologous regions of NA7 Nef and are represented by open bars. The locations of the N-terminal (N-term), central (Central) and C-terminal (C-term) segments of HIV-1 and SIV Nef analyzed in detail in this work are indicated at the bottom of the figure.

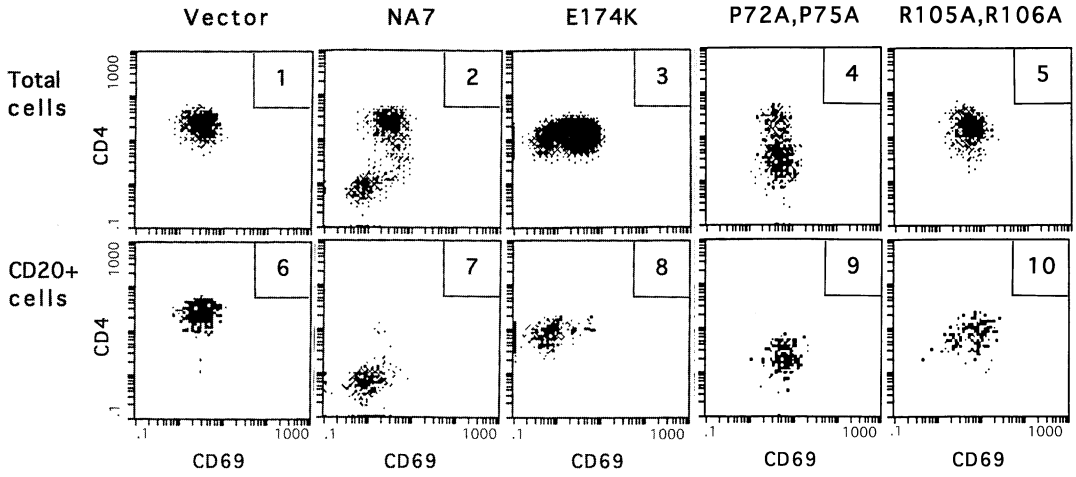


Fig. 4. Mutations separate the effect of Nef on CD4 and on CD69 expression. Analysis of CD4 and CD69 expression in Jurkat T cells transfected with control plasmids (Vector, NA7, panels 1, 2, 6 and 7) or with plasmids expressing selected mutant HIV-1 Nef proteins (panels 3–5 and 8–10). Amino acid substitutions are indicated above the panels (E174K, P72A, P75A, R105A and R106A). CD4 and CD69 expression on total surviving cells (Total cells) and on CD20-positive cells ($CD20^+$ cells) is shown.

signaling, having only a minor effect on CD4 down-regulation (Figure 4, compare panel 4 with 1 and 2 and panel 9 with 6 and 7). Both of these observations indicate that the effects on CD4 and CD69 expression are separate functions of Nef. Mutations of the third class, exemplified by alanine substitutions for R105 and R106, severely compromised both the effect of Nef on CD4 expression and on CD3 signaling (Figure 4, compare panel 5 with 1 and 2 and panel 10 with 6 and 7). Mutations of the final class did not alter the effect of Nef on CD4 and CD69 expression.

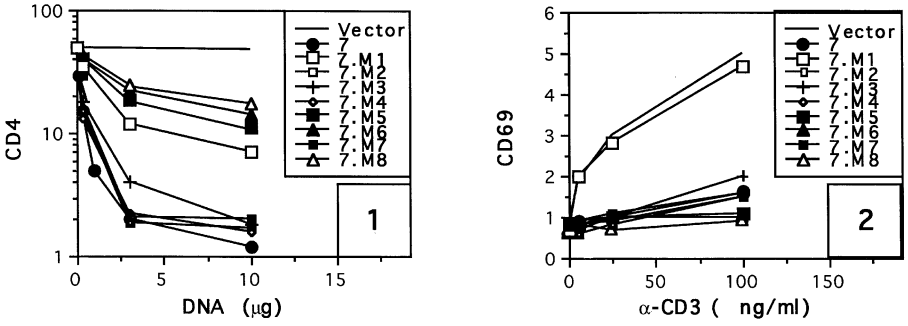
Mutations in the N-terminal region of HIV-1 Nef selectively disrupt CD4 down-regulation

Mutations which selectively disrupt the effect of Nef on CD4 expression were located in two regions of the Nef molecule. These regions were analyzed separately in Figures 5 and 6. As shown in Figure 5A, one such region was defined by analyzing mutants in the 57 residue N-terminal segment of HIV-1 NA7 Nef (mutants 7.M1–7.M8; see also Figure 3 for the definition of the N-terminal region). The mutation in 7.M1 Nef disrupts the N-terminal myristoylation signal and is known to diminish the activity of the viral protein in several cellular and viral assays (Mariani and Skowronski, 1993; Aiken *et al.*, 1994; Aiken and Trono, 1995). Mutations in 7.M2–7.M8 Nef were designed to disrupt the structure of the N-terminal region by substituting alanine for charged residues or aspartate for glycines or alanines. The effect of mutant Nef proteins on CD4 expression was quantitated in dose–response experiments, which allowed a reliable comparison of wild-type and mutant Nef proteins. As shown in Figure 5A, the mutations in 7.M1, 7.M5, 7.M6 and 7.M8 severely impaired the ability of Nef to down-regulate CD4 expression (Figure 5A, panel 1). Analysis of the dose–response curves showed that for the wild-type NA7 plasmid, the half-maximal down-regulation of CD4 expression required 0.15 μg DNA. In contrast, with the 7.M8 plasmid the same effect required ~20 times more DNA (defined as 5% of wild-type activity; see Figure 5A). The 7.M2 and 7.M7 mutants were the same as the wild-type in their ability to down-regulate CD4 expression.

The ability of the mutant Nef proteins to block CD69 induction by anti-CD3 MAb was assessed by dose–

A

Name	Mutation	1 ... 57	Downregulation of expression: CD4	CD69
NA7		MGGKWSKRSAGGWSAVRKRMEQAEPAADGVGAVSRDLEKYGAITSSNTATNNADCAW	100	+++
7.M1	2A	.A.....	15	-
7.M2	4A,7AA	...A..AA.....	50	+++
7.M3	17AAA,21A	.....AAA.A.....	100	+++
7.M4	24A,28A	.....A...A.....	100	+++
7.M5	29R,36G	.....R.....G.....	8	+++
7.M6	35AA	.....AA.....	6	+++
7.M7	38AA	.....AA.....	40	+++
7.M8	56D	.....D.	5	+++



B

Name	Mutation	50 ... 88	Downregulation of expression: CD4	CD69
239		LSSLSCEGQKYNQGQYMNTPWRNPAEEREKLAYRKQNMD	100	+++
239.M1	54AAA	....AAA.....	62	+++
239.M2	66ED	.....ED.....	6	+++
239.M3	69D	.....D.....	5	+++
239.M4	73ED	.....ED.....	11	+++
239.M5	79A	.....A.....	60	+++

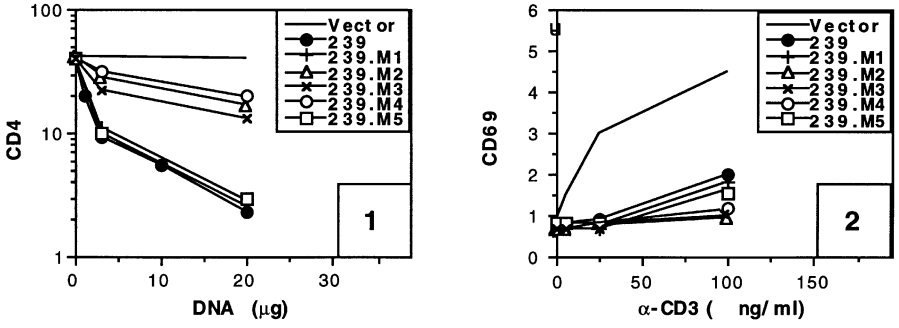


Fig. 5. Mutations in the N-terminal segment of Nef selectively disrupt the effect on CD4 expression. (**A**) Amino acid sequences of a set of mutant NA7 Nef proteins are aligned with that of wild-type NA7 Nef. Dots indicate amino acid identity with the wild-type protein and letters identify amino acid substitutions in the single letter code. The nomenclature of the mutant Nef proteins and of the corresponding mutations are defined on the left side of the panel. The relative ability of the wild-type and mutant Nef proteins to down-regulate CD4 expression and to block induction of CD69 by anti-CD3 MAb are shown on the right. The relative ability of *nef* alleles to down-regulate CD4 expression was calculated as the ratio of the amount of the vector encoding the wild-type Nef required for half-maximal reduction of CD4 expression to that of a vector encoding mutant Nef protein required for a similar effect, calculated from dose–response experiments performed as shown in panel 1 below. The ability of Nef proteins to block CD69 induction by anti-CD3 MAb is indicated by +++, ++, +, +/– or –, which reflect blocking of CD69 induction in response to 100, 25 and 5 ng/ml, a partial blocking at 5 ng/ml or no blocking at any of the tested concentrations of anti-CD3 MAb, determined as shown in panel 2 below. (Panel 1) Dose–response analysis of the effect of mutant NA7 Nef proteins on CD4 expression is shown. CD4 expression on CD20-positive cells is shown on the ordinate as peak channel number of CD4 fluorescence. The amount of vector DNA used for transfection is shown on the abscissa. (Panel 2) Induction of CD69 expression by increasing doses of anti-CD3 MAb is shown. Jurkat T cells were co-electroporated with 20 μg vector expressing wild-type or mutant Nef proteins and 3 μg CD20 reporter plasmid and cultured in the presence of the indicated concentrations of HIT-3A anti-CD3 MAb (0, 5, 25 and 100 ng/ml). CD4, CD69 and CD20 expression were detected simultaneously by three color flow cytometry. CD69 expression on the surface of CD20-positive cells stimulated overnight with the indicated amounts of anti-CD3 MAb is shown on the ordinate as peak channel number of CD69 fluorescence. Each data set is representative of three determinations. In all experiments wild-type NA7 *nef* (NA7) was used as a positive control and an empty vector (Vector) was used as a negative control. The error in CD69 expression measurement was ±0.5 units and that in CD4 expression measurement was ±25%. (**B**) Results from the analysis of mutations in the N-terminal region of mac239 Nef are shown as described above for the NA7 Nef mutants.

response analysis, which allowed a reliable quantitation of this effect of Nef. In these experiments, the induction of CD69 surface expression was measured in cells transfected with 20 μg vector expressing the wild-type or mutant Nef protein and exposed to increasing doses of anti-CD3 MAb (Figure 5A, panel 2). The wild-type NA7 Nef protein

A

Name	Mutation				Downregulation of expression: CD4	CD69
7.M19	174AA	.....AA.....			<2	+++
7.M20	174A	.....A.....			8	+++
7.M21	174K	.....K.....			<2	+++
7.M22	175A	.....A.....			4	+++
7.M23	177AAA	.....AAA.....			<2	+++
NA7		164	LLHPMSLHGMEDAEREVLQWKFDSRLA	190	100	+++
239		194	LMHPAQTSQWDDPWGEVLAWKFDPTLA	220	100	+++
239.M10	204AA	.....AA.....			<5	+
239.M11	204RR	.....RR.....			<5	+++
239.M12	204K	.....K.....			<5	+++
239.M13	207AAA	.....AAA.....			<5	+

B

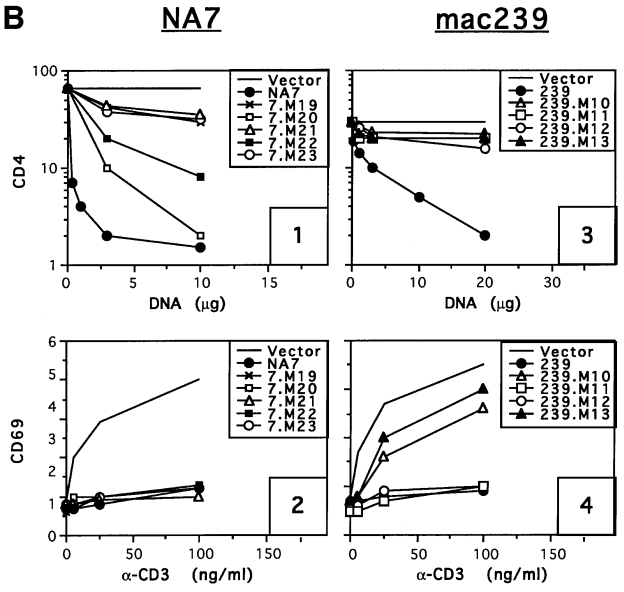


Fig. 6. Mutations in the C-terminal region of Nef selectively disrupt down-regulation of CD4 expression. (**A**) A compilation of the effect of mutations in the C-terminal region of Nef on CD4 down-regulation and on the blocking of CD69 induction in response to anti-CD3 MAb is shown. (**B**) Dose–response analysis of the effect of mutant NA7 (NA7, panels 1 and 2) and mac239 (mac239, panels 3 and 4) Nef proteins on CD4 (panels 1 and 3) and CD69 (panels 2 and 4) expression was performed as described in Figure 5.

blocked induction of CD69 expression for all three concentrations of anti-CD3 MAb tested (Figure 5A, compare NA7 and Vector in panel 1). In contrast, the myristoylation-defective 7.M1 Nef did not block CD69 expression, even at the lowest dose of anti-CD3 MAb. Therefore, an intact myristoylation signal is required for Nef to block CD3 signaling. The remaining mutant Nef proteins blocked CD69 expression as well as the wild-type protein (the slight variation in CD69 expression level observed with these proteins following stimulation of cells with 100 ng/ml anti-CD3 MAb was within the limits of reproducibility of the assay). Thus, the amino acid residues mutated in the 7.M5, 7.M6 and 7.M8 Nef proteins define the N-terminal region of HIV-1 Nef that is required for the ability of Nef to down-regulate CD4 expression.

Mutations in the N-terminal region of SIV Nef also selectively disrupt CD4 down-regulation

The amino acid sequence of the N-terminal region of HIV-1 Nef important for CD4 down-regulation is poorly conserved between HIV-1 and SIV proteins (see Figure 3; Myers *et al.*, 1993). However, the HIV-1 and SIV proteins can both down-regulate CD4 expression. To address whether the N-terminal regions of HIV-1 and SIV Nef are functionally homologous we tested the effect of mutations in the N-terminal portions of the mac239 Nef molecule on CD4 down-regulation and on blocking of CD69 induction by anti-CD3 MAb.

As shown in Figure 5B, three mutations designed to disrupt the organization of the N-terminal region in mac239 Nef, by substituting alanine, proline and methionine residues with charged amino acids, severely reduced CD4 down-regulation by Nef yet did not have a detectable effect on the ability of Nef to block CD69 induction (Figure 5B, see 239.M2, 239.M3 and 239.M4). Mutations in 239.M1 and 239.M5 did not have detectable effects on the activities of Nef. Thus, despite dissimilar amino acid sequences, the N-terminal regions of NA7 and mac239 Nef proteins are functionally homologous.

Mutations in the C-terminal region of HIV-1 and SIV Nef also selectively disrupt CD4 down-regulation

The second group of mutations that selectively disrupted CD4 down-regulation are located in the C-terminal part of the core of the NA7 molecule (see Figures 3 and 6). A double alanine substitution at positions 174 and 175 in 7.M19 reduced by >50-fold the ability of Nef to down-regulate CD4 expression (Figure 6, 7.M19; in agreement with Aiken *et al.*, 1996). As can be seen from the dose–response curves obtained with mutants 7.M20 and 7.M22, each of the two individual alanine substitutions contributed to the defect (Figure 6B, panel 1). In addition, a lysine substitution at position 174 (7.M21) completely disrupted CD4 down-regulation. A triple alanine substitution for three adjacent charged amino acids had a comparably

A

Name	Mutation		Downregulation of expression: CD4	CD69	Nef protein stability (% wt)
		"--" "PxxP" "PKC" "KEK" "RR"			
7.M9	62AAAA	.....AAAA.....	50	-	105
7.M10	72A,75A	.....A..A.....	50	-	85
7.M11	77A	.....A.....	40	++	35
7.M12	82A	.....A.....	70	+++	85
7.M13	86A	.....A.....	50	-	100
7.M14	80A	.....A.....	40	++	20
7.M15	92AAA	.....AAA.....	60	++	140
7.M16	105AA	.....AA.....	5	-	25
7.M17	105A	.....A.....	30	+	(ND)
7.M18	106A	.....A.....	30	-	(ND)
NA7		55 CAWLEAQEEEEVGFPVRPQVPLRPMTYKAAVDISHFLKEKGGLEGLIHSQRRQDILDL 112	100	+++	100
239		87 MDDIDEEDDDLVGVSVRPKVPLRTMSYKLAIDMSHFIKEKGGLEGIYYSARRHRILDI 134	100	+++	100
239.M6	88NN,91NQQNNN	.NN.NQQNNN.....	<<5	-/+	20
239.M7	104A,107A	.....A..A.....	60	++	25
239.M8	110A,112A	.....A.A.....	30	-/+	15
239.M9	127AA	.....AA.....	5	-	10

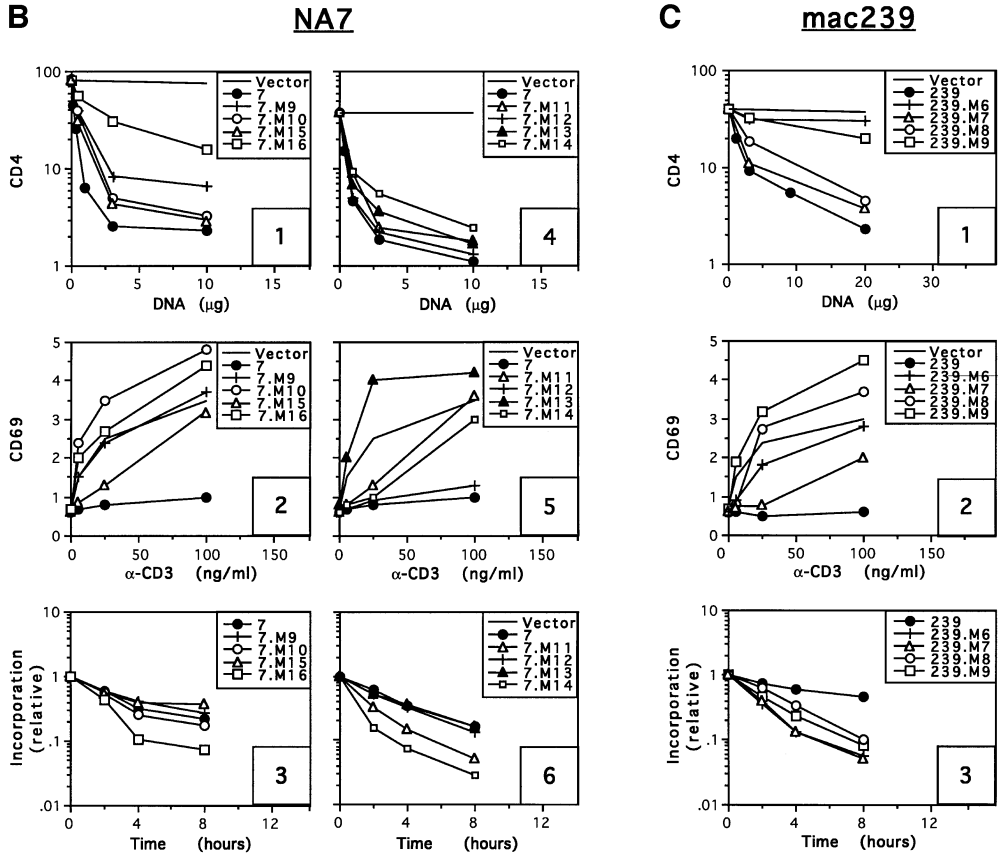


Fig. 7. Mutations in the central region of the Nef molecule preferentially disrupt the effect on CD3 signaling. (**A**) Compilation of the effect of mutations in the central region of NA7 and mac239 Nef proteins on CD4 down-regulation and on the blocking of CD69 induction in response to anti-CD3 MAb is shown. ND, not determined. (**B**) Dose–response analysis of the effect of mutant NA7 Nef proteins on CD4 expression (panels 1 and 4) and on CD69 expression (panels 2 and 5) was performed as described in Figure 5. The relative stability of the wild-type and mutant Nef proteins was determined as described in Materials and methods (panels 3 and 6). (**C**) Dose–response analysis (panels 1 and 2) and relative stabilities (panel 3) of mutant mac239 Nef proteins are shown.

detrimental effect on CD4 down-regulation (7.M23). As shown in Figure 6B, none of these mutations affected the ability of Nef to block CD69 induction (see 7.M19–7.M23 in panel 2). These results defined a second region of Nef that is required for CD4 down-regulation but not for blocking CD69 induction.

We tested whether the corresponding region of SIV Nef was similarly involved in CD4 down-regulation. The design of mutations made in the C-terminal region of mac239 Nef is shown in Figure 6A. Initially, double and triple alanine substitutions (mutants 239.M10 and 239.M13), similar to those mutations that disrupted CD4 down-regulation in NA7 Nef (mutants 7.M19 and 7.M23), were tested (Figure 6A). As shown in Figure 6B, both mutations not only severely impaired the ability of mac239 Nef to down-regulate CD4 expression, but also disrupted its effect on the induction of CD69 expression (see panels 3 and 4 respectively), and both destabilized the Nef protein

(data not shown). However, substitutions of charged amino acids at the same positions in mutants 239.M11 and 239.M12 resulted in proteins that were selectively defective for down-regulation of CD4 surface expression (Figure 6B, see 239.M11, 239.M12 in panels 3 and 4). Therefore, the corresponding C-terminal regions of HIV-1 and SIV Nef are both required for CD4 down-regulation.

Mutations in the HIV-1 Nef core preferentially disrupt the effect on CD3 signaling

The conserved Nef core has been shown to associate with SH3 domains of Src family protein tyrosine kinases and with a PAK family serine kinase (Sawai *et al.*, 1994; Saksela *et al.*, 1995; Nunn and Marsh, 1996). To assess the role of the Nef core in the down-regulation of CD4 expression and in the blocking of CD3 signaling, we assayed the effects of mutations that disrupted the conserved sequence elements in the central region of Nef (Shugars *et al.*, 1993; see Figure 7A).

Notably, three of the mutant alleles, 7.M9, 7.M10 and 7.M13, resulted in Nef proteins that were unable to block induction of CD69 expression even by the lowest amount of anti-CD3 MAb tested (Figure 7B, see 7.M9, 7.M10 in panel 2 and 7.M13 in panel 5). The mutation in 7.M9 Nef disrupted a stretch of negatively charged amino acid residues adjacent to the Nef core. Each of the remaining two mutations disrupted separate aspects of the SH3 binding surface in the Nef core: P72 and P75 of the PPII helix (7.M10) and R86 (7.M13), both of which contribute to the high affinity Nef–SH3 domain interaction (Lee *et al.*, 1996). Remarkably, none of the three mutations had a significant effect on the ability of Nef to down-regulate CD4 expression (Figure 7B, see panels 1 and 4) or on the stability of Nef as measured by pulse chase analysis (see panels 3 and 6). Thus these mutations likely resulted in properly folded Nef proteins that were deficient in a specific interaction(s). In contrast, substituting alanines for R105 and R106 (Figure 7B, see 7.M16), which are required for the association of Nef with the p62 serine kinase activity (Sawai *et al.*, 1994), resulted in an unstable protein that was severely impaired for down-regulation of both CD4 and CD69 expression (see 7.M16). The alanine substitution for T80 in 7.M14 Nef had a similar, but less pronounced, phenotype. The remaining mutations did not result in overt phenotypes (Figure 7B, see 7.M11, 7.M12 and 7.M15). Thus, the central portion of the HIV-1 Nef molecule, which includes residues involved in SH3 interactions, is critical for the ability of Nef to block CD3 signaling. Moreover, these results demonstrate again that the effects of Nef on CD4 surface expression and CD3 signaling are separate.

To assess whether our observations from HIV-1 Nef also apply to SIV Nef, the effects of selected mutations in the mac239 Nef core on CD4 and CD69 expression were studied (Figure 7A). Three mutations that disrupted the acidic region (239.M6), substituted alanine residues for T110 and S112 (239.M8) or substituted alanine residues for R127 and R128 (239.M9), which are required for association of mac239 Nef with the p62 serine kinase activity, were tested (Sawai *et al.*, 1995). These mutations resulted in unstable proteins that were defective in both assays (see Figure 7C).

Interestingly, mutating P104 and P107 of the PxxP motif in 239.M7 had only a partially disruptive effect on the ability of Nef to block CD69 induction by anti-CD3 MAb (see 239.M7 in Figure 7A and C). This result is in contrast to our observations with the PxxP motif mutation in NA7 Nef (see 7.M10 in Figure 7). Thus, the NA7 and mac239 Nef cores are organized differently and some residues in addition to P104 and P107 are responsible for the effect of mac239 Nef on CD3 signaling.

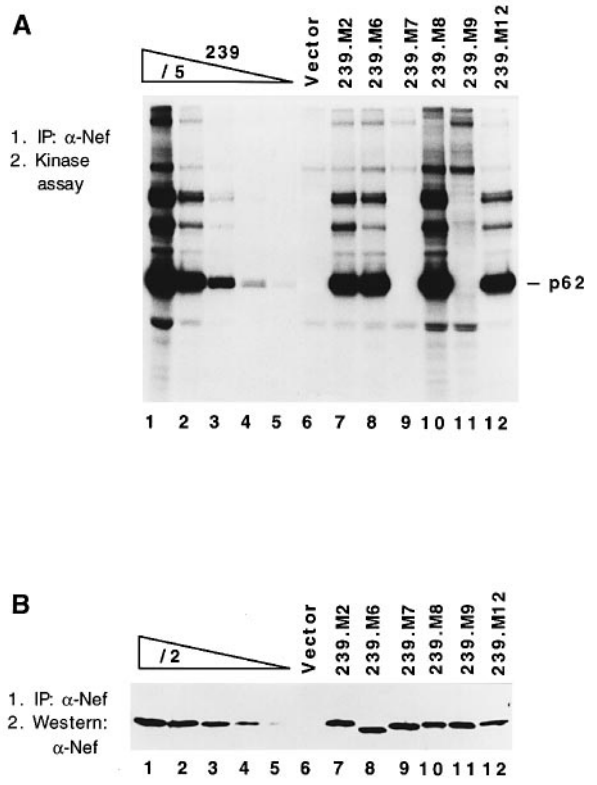


Fig. 8. Effect of mutations in mac239 Nef on the association with p62 protein kinase. (**A**) *In vitro* kinase assays were performed with Nef immune complexes prepared from Cos-7 cells transfected with plasmids expressing mac239 *nef* (239, lanes 1–5), selected mutant mac239 *nef* alleles (239.M2–239.M15, lanes 7–12) or control empty vector (Vector, lane 6). All Nef immune complex reactions were normalized to contain equal amounts of the wild-type and mutant Nef proteins. Five-fold serial dilutions of the kinase reaction performed with the wild-type mac239 Nef immune complex were used as standards for quantitation (lanes 1–5). (**B**) Aliquots of the normalized Nef immune complex reactions shown in (A) were immunoblotted with the rabbit anti-Nef serum. Two-fold serial dilutions of the wild-type mac239 Nef immune complex were used as standards for quantitation (lanes 1–5).

The effect of Nef on CD69 surface expression does not correlate with the association of Nef with the p62 serine kinase activity

Nef associates with a p62 serine kinase activity and this interaction may be important for its effect on signal transduction in T cells (Baur *et al.*, 1994). To assess whether this association is important for the effect of Nef on CD3 signaling and CD4 surface expression, we tested the ability of selected mutant mac239 Nef proteins to associate with the p62 kinase activity in a co-immunoprecipitation assay. As shown in Figure 8, neither the mutations in 239.M2 and 239.M12, which disrupted the ability of Nef to down-regulate CD4 expression, nor those in 239.M6 and 239.M8, which compromised the ability

of Nef to block CD69 induction, had a detectable effect on the association of Nef with the p62 phosphoprotein nor with other phosphoproteins found associated with wild-type mac239 Nef (Figure 8, compare lanes 7, 8, 10 and 12 with 2). Interestingly, the PxxP motif mutation (239.M7), which reduced the association of Nef with the p62 phosphoprotein by >500-fold (Figure 8, compare lane 9 with standards in 1–5), had only a small effect on the ability of Nef to modulate CD4 or CD69 expression (Figure 7A and C, 239.M7). The lack of correlation between the effect of Nef on CD4 and CD69 expression and Nef–p62 association indicates that some other molecular interaction(s) is limiting for the effects of Nef on CD4 expression and CD3 signaling.

Discussion

We used a transient expression assay to study the interactions of the HIV-1 and SIV Nef proteins with the TCR machinery in a human $CD4^+$ Jurkat T cell line. We have demonstrated that natural HIV-1 and SIV Nef proteins block a subset of signals emanating from the TCR, as measured by a block to the induction of CD69 expression by anti-CD3 MAb. This observation is in agreement with previous data indicating that stable expression of the HIV-1 Nef protein in Jurkat T cells can interfere with induction of IL-2 gene expression by lectins and by anti-CD3 MAb (Luria *et al.*, 1991; Baur *et al.*, 1994; Collette *et al.*, 1995). However, since the Nef-mediated block to CD69 induction was bypassed by a calcium ionophore while the previously described Nef-induced block to the induction of IL-2 expression was not overcome by a calcium ionophore, it appears that the transient assay permitted us to measure new aspects of Nef function. This assay also allowed us to circumvent the possible selection for variants in signal transduction pathways in constitutively expressing Nef cell lines (Luria *et al.*, 1991; Baur *et al.*, 1994).

Previous work has described Nef-induced down-regulation of CD4 and concomitant disruption of the CD4–Lck complex (Aiken *et al.*, 1994; Anderson *et al.*, 1994; Salghetti *et al.*, 1995). Results from our mutational analysis of the HIV-1 and SIV Nef proteins indicate that the effects of Nef on CD4 expression and on CD3 signaling are separate functions of the viral protein.

We predict that the net effect of Nef on antigen-specific TCR signaling will be combinatorial. It will be determined by the ability of Nef to disrupt CD3 signaling as well as by the ability of Nef to down-regulate CD4 expression and will depend on the amount of Nef expressed. This is perhaps best illustrated by the contrasting effects of Nef expression in Jurkat T cells, where Nef blocks CD3 signaling, and in thymocytes in transgenic mice, where CD3-dependent responses are enhanced (Skowronski *et al.*, 1993; Lindemann *et al.*, 1994). The positive effect of Nef on T cell activation in transgenic mice appears to require the CD4 molecule and may reflect the release of Lck from a complex with CD4 (Haughn *et al.*, 1992; M.Lock, D.R.Littman and J.Skowronski, unpublished results). The level of Nef expression in T cells from the transgenic mice was lower than that required for blocking CD69 induction in transiently transfected Jurkat T cells (M.Lock and J.Skowronski, unpublished results). The differential contribution of CD4-dependent and CD4-independent effects may explain the different consequences of Nef expression on CD3 signaling in $CD4^+$ Jurkat T cells and in $CD4^+$ T lymphocytes from transgenic mice.

Our studies provide evidence for the molecular interactions that underlie the effect of Nef on CD3 signaling and on CD4 down-regulation. The interaction(s) that is required for the ability of Nef to block CD3 signaling was selectively compromised by mutations in the conserved core of the HIV-1 Nef protein. The most drastic effect was with the 7.M10 Nef mutant, which contains alanine substitutions for P72 and P75 in the PxxP motif, and with the 7.M13 Nef mutant, which contains an alanine substitution for D86 (see Figure 7A). Recent results from NMR and crystal structure analyses of Nef–SH3 domain complexes indicated that these three amino acid residues form part of the SH3 binding surface of Nef and are important for high affinity specific interaction with Src family SH3 domains (Grzesiek *et al.*, 1996; Lee *et al.*, 1996). The P72 and P75 residues are the critical components of the PPII helix of Nef that make contacts with the SH3 domain core. The D86 residue contacts the RT loop of the SH3 domain. This latter interaction determines the specificity of Nef for a subset of Src family SH3 domains and stabilizes the overall interaction (Grzesiek *et al.*, 1996; Lee *et al.*, 1996). Thus, our observations establish a clear link between the SH3 binding surface of HIV-1 Nef and its ability to block CD3 signaling.

Mutating the PxxP motif in the PPII helix in mac239 Nef had a much less drastic effect on CD3 blocking than the corresponding mutation in NA7 Nef (compare 7.M10 in Figure 7B with 239.M7 in Figure 7C). However, it is evident from the structural studies that, in addition to the PPII helix, other elements in the tertiary structure of the folded Nef molecule are required for high affinity interaction with SH3 domains (Lee *et al.*, 1996). Therefore, it is possible that these additional stabilizing interactions are sufficient to support a functional, although weakened, interaction between 239.M7 and a target SH3 domain. However, it is also possible that another non-SH3 interaction is important for the effect of mac239 Nef on CD3 signaling.

Three cellular proteins, a p62 PAK family serine kinase and the Hck and Lyn Src family tyrosine kinases, have been shown to associate with the Nef core (Sawai *et al.*, 1994; Saksela *et al.*, 1995). Several observations argue against the importance of these associations for the effect of Nef on TCR signaling. First, although the p62 serine kinase activity associates efficiently with mac239 Nef and with Nef proteins from certain HIV-1 isolates (Sawai *et al.*, 1995; Luo and Garcia, 1996), it is evident from our genetic and biochemical experiments that this association does not correlate with the ability of Nef to disrupt TCR signaling. Second, Hck is found predominantly in cells from the monocyte/macrophage lineage and is not significantly expressed in T cells (Brickell, 1992). Third, neither Hck nor Lyn were found to be important for signal transduction in T cells (Lowell *et al.*, 1994; Nishizumi *et al.*, 1995). Thus, interactions between the core region of Nef and other cellular targets must exist which explain the effect of Nef on signaling in T cells.

The ability of Nef to down-regulate CD4 expression

was severely and selectively disrupted by mutations in two regions of the NA7 Nef molecule. These regions, located between amino acid residues 36 and 56 in the N-terminal part of NA7 Nef and between residues 174 and 179 in the C-terminal part of NA7 Nef, are not well conserved between the NA7 Nef and mac239 Nef proteins (see Figure 3). Nevertheless, mutations in the homologous regions in mac239 Nef also disrupted CD4 down-regulation. Thus, in respect of CD4 down-regulation, the HIV-1 and SIV proteins have the same general functional organization.

It is interesting that the N- and C-terminal regions of NA7 Nef that are important for CD4 down-regulation correspond to two flexible loops, as determined from structural studies (Grzesiek *et al.*, 1996; Lee *et al.*, 1996). This suggests a model in which these two loops engage in two or more distinct interactions with the CD4 molecule itself and/or with other components of the endocytotic machinery. While the similar functions and the similar functional organization of the mac239 and NA7 Nef proteins argue that they interact with the same cellular targets to down-regulate CD4 expression, the dissimilarity of amino acid sequences of the flexible loops in SIV and HIV-1 Nef suggests that each protein makes different contacts with the same cellular targets.

The abilities of Nef to alter CD4 expression and to alter CD3 signaling are conserved with natural Nef proteins derived from HIV-1-infected individuals and from AIDS patients (Anderson *et al.*, 1993; Mariani and Skowronski, 1993; Iafrate and Skowronski, data not shown) and therefore are clearly important for viral replication *in vivo* and for pathogenesis. While the roles of these two effects of Nef in the biology of immunodeficiency viruses are not yet known, we speculate that they may reflect (i) redirecting of certain key components of the T cell signal transduction machinery by Nef to enhance viral replication and/or (ii) a coordinated effort to alter the normal pattern of T cell responses to the advantage of the virus. In particular, modulation of TCR signaling by Nef could alter a range of T cell responses, including functional activation and programed cell death, both of which could play important roles in the immunodeficiency virus life cycle *in vivo*.

Materials and methods

Plasmid construction

The protein coding region of the natural HIV-1 NA7 *nef* (Mariani and Skowronski, 1993) and of SIV mac239 'open' *nef* (Regier and Desrosiers, 1993; kindly provided by R.C.Desrosiers) were subcloned between the *Xba*I and *Bam*HI sites of plasmid pBS(–) (Stratagene) and oligonucleotide-directed site-specific mutagenesis was performed as previously described (Mariani and Skowronski, 1993). The mutated *nef* sequences were verified by DNA sequencing and subcloned between the *Xba*I and *Bam*HI sites of a T cell-specific pCD3-β expression vector, as previously described (Skowronski *et al.*, 1993). The pCG and pCGT vectors (Tanaka and Herr, 1990; Wilson *et al.*, 1995), containing constitutively active forms of Ras, Rac or Raf under the control of the CMV promoter, were provided by Linda van Aelst. Murine Lck cDNA, provided by Dan R.Littman, was subcloned into the pCD3-β and pCG expression vectors using standard techniques (Sambrook *et al.*, 1989).

DNA transfections and stimulation of Jurkat T cells

Jurkat T cells (JJK) expressing human CD4 at a high level, provided by Dan R.Littman, were maintained in RPMI 1640 medium supplemented with 2 mM glutamine and 20 mM HEPES, pH 7.4, and cultures were diluted 1:10–1:20 every 3–4 days. Aliquots of 10^7 cells from exponentially growing cultures were electroporated at 200 V and 960 μF with a total of 10–20 μg DNA containing varying amounts of appropriate expression vectors and 3 μg CMV CD20 expression plasmid for use as a marker of transfected cells (Zhu *et al*., 1993). The amount of CMV CD20 reporter plasmid used in co-transfection experiments was kept relatively low to ensure that the CD20 reporter was co-expressed with the appropriate effector protein in the majority of $CD20^+$ cells. Approximately 24 h following electroporation cells were stimulated by the addition of HIT3A MAb (PharMingen), specific for the ε subunit of CD3, or by the addition of phorbol myristate acetate (PMA) or a calcium ionophore (A23187) (Calbiochem) and cultured for an additional 12–15 h prior to flow cytometric analysis of CD4 and/or CD69 expression. All stimulations were dose–response experiments.

Flow cytometric analysis of CD4 and CD69 expression

Aliquots of 2×10^5–4×10^5 Jurkat T cells were washed once with phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS) and 0.1% sodium azide (PBS-FA). Cells were reacted in the wells of a 96-well microtiter plate at 4°C with 50 μl aliquots of a cocktail containing saturating amounts of phycoerythrin-conjugated Leu3A MAb, specific for CD4 (Becton and Dickinson), PerCP-conjugated Leu-16 MAb, specific for CD20 (Becton and Dickinson), and fluorescein-conjugated FN50 MAb, specific for CD69 (PharMingen). Cells were then washed twice with PBS-FA, suspended in 200 μl PBS-FA and expression of CD4, CD69 and CD20 molecules on the cell surface was analyzed on an Epics-Elite flow cytometer. Dead cells and cell debris (usually 20–40% of total events) were excluded from the analysis by bitmap gating on forward scatter and 90° scatter profiles. In a typical experiment ~5–15% of cells that survived electroporation showed readily detectable expression of the CD20 surface marker. For dose–response analysis (Mariani and Skowronski, 1993; Skowronski and Mariani, 1995), the level of CD4 or CD69 expression on the surface of CD20-positive cells, represented by peak channel number of red or green fluorescence, was measured as a function of the amount of Nef expression vector DNA used for transfections (CD4) or as a function of the amount of activating agent used in T cell activation experiments (CD69).

Immune complex kinase assays

Cos-7 cells (Gluzman, 1981) were electroporated at 200 V and 960 μF in 250 μl of solution containing 10 μg pCG based Nef-expression vector or a control plasmid, 50 μl 150 mM NaCl and 200 μl Dulbecco's modified Eagle's medium containing 10% FBS and 20 mM HEPES, pH 7.4. Approximately 15–20 h post-transfection cells were lysed for 40 min on ice in buffer containing 1% Triton X-100, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.1 mM sodium vanadate and 0.5 mM sodium fluoride. Nuclei and cell debris were removed by centrifugation at 10 000 *g* for 10 min at 4°C. The cytoplasmic extracts were precleared with protein G–agarose beads (Boehringer-Mannheim) for 30 min at 4°C and incubated with 2 μl anti-mac239 Nef rabbit serum (provided by F.Kirchhoff and R.C.Desrosiers) for 1 h at 4°C. Immune complexes were then bound to protein G–agarose beads for 40 min at 4°C. The beads were then washed three times with lysis buffer, twice with lysis buffer without glycerol and once with kinase reaction buffer containing 50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100 and 0.1 mM sodium vanadate (Sawai *et al.*, 1994). For the immune complex kinase assay, the beads were suspended in 50 μl ice-cold kinase reaction buffer containing 30 μCi [γ-^{32}P]ATP (7000 Ci/mmol; ICN) and the reaction was carried out for 5 min at room temperature. The reaction was stopped by addition of 500 μl ice-cold kinase reaction buffer containing 5 mM EDTA and 2 mM sodium vanadate and the beads were then washed twice with this solution. The washed beads were suspended in SDS sample buffer and the protein complexes were recovered following 10 min incubation at 80°C. To evaluate the kinase activity present in the Nef immune complexes, aliquots of immune complexes were resolved on 9% polyacrylamide gels and dried gels were exposed to X-ray film and/or subjected to phosphorimager analysis (BAS-1000, FUJI).

Immunoblot analysis of Nef expression

Cytoplasmic extracts from transiently transfected Cos-7 cells were prepared as described above. Aliquots of immune complexes prepared from Cos-7 cells were denatured in reducing sample buffer, resolved on 14% polyacrylamide gels and electroblotted onto poly(vinylidene difluoride) membrane (Immobilon; Millipore) as described previously (Salghetti *et al.*, 1995). Membranes were incubated for 1 h in blocking solution containing NT buffer [150 mM NaCl, 50 mM Tris–HCl, pH 7.5,

with 0.1% Tween 20, 1% bovine serum albumin and 5% non-fat dried milk (Carnation)] and then exposed to anti-mac239 SIV Nef serum (1:1000 dilution) in the blocking solution. Membranes were washed three times with NT buffer containing 0.1% Tween 20, incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies and developed using the ECL detection system under the conditions recommended by the manufacturer (Amersham).

Determination of Nef protein stability

Cos-7 cells were co-transfected by electroporation with pCG vectors expressing the wild-type or mutant alleles of NA7 or mac239 *nef* along with a reporter plasmid encoding a mutant CD4d402 protein, which lacks the cytoplasmic domain and whose expression and stability is not altered in the presence of Nef (Aiken *et al.*, 1994; De and Marsh, 1994; Salghetti *et al.*, 1995), and each transfection was aliquoted into 6-well microtiter plates. Following overnight incubation, cells were metabolically labeled for 4 h in the presence of 20 μCi Trans-[^{35}S]-label (ICN). After labeling, cells were washed and chased for 0, 2, 4 or 8 h. Subsequently, cytoplasmic extracts prepared from radiolabeled cells were reacted with a mixture containing an excess of anti-Nef serum and anti-CD4 MAb OKT4 and the Nef and CD4 immune complexes were isolated with protein G beads, as described above. Immune complexes were resolved on 15% polyacrylamide gels and ^{35}S incorporation into Nef and CD4d402 was quantitated by phosphorimager (BAS-1000, FUJI).

Acknowledgements

We thank Ron C.Desrosiers, Frank Kirchhoff and Linda van Aelst who kindly provided various reagents used in this work, Roberto Mariani for help in the mutagenesis of HIV-1 *nef* at early stages of this project and Linda van Aelst, William Tansey and members of the laboratory for discussions. We are indebted to Pat Burfeind for help and for creative assistance with flow cytometry analysis. This work was supported by grants from the Public Health Service (IA-35394), from the Johnson & Johnson Focused Giving Program and from Cold Spring Harbor Laboratory funds.

References

Aiken,C. and Trono,D. (1995) Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J. Virol*., **69**, 5048–5056.

Aiken,C., Konner,J., Landau,N.R., Lenburg,M.E. and Trono,D. (1994) Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell*, **76**, 853–864.

Aiken,C., Krause,L., Chen,Y.L. and Trono,D. (1996) Mutational analysis of HIV-1 Nef: identification of two mutants that are temperature-sensitive for CD4 downregulation. *Virology*, **217**, 293–300.

Anderson,S., Shugars,D.C., Swanstrom,R. and Garcia,J.V. (1993) Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. *J. Virol*., **67**, 4923–4931.

Anderson,S.J., Lenburg,M., Landau,N.R. and Garcia,J.V. (1994) The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. *J. Virol*., **68**, 3092–3101.

Baur,A.S., Sawai,E.T., Dazin,P., Fantl,W.J., Cheng-Mayer,C. and Peterlin,B.M. (1994) HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity*, **1**, 373–384.

Benson,R.E., Sanfridson,A., Ottinger,J.S., Doyle,C. and Cullen,B.R. (1993) Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *J. Exp. Med*., **177**, 1561–1566.

Brady,H.J., Pennington,D.J., Miles,C.G. and Dzierzak,E.A. (1993) CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration. *EMBO J*., **12**, 4923–4932.

Brickell,P.M. (1992) The p60c-src family of protein-tyrosine kinases: structure, regulation and function. *Crit. Rev. Oncogen*., **3**, 401–446.

Collette,Y., Chang,H.L., Cerdan,C., Chambot,H., Algarte,M., Mawas,C., Imbert,J., Burny,A. and Olive,D. (1995) Specific Th1 cytokine downregulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression. *J. Immunol*., **156**, 360–370.

De,S.K. and Marsh,J.W. (1994) HIV-1 Nef inhibits a common activation pathway in NIH3T3 cells. *J. Biol. Chem*., **269**, 6656–6660.

de Ronde,A., Klaver,B., Keulen,W., Smit,L. and Goudsmit,J. (1992) Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. *Virology*, **188**, 391–395.

Deacon,N.J. *et al.* (1995) Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science*, **270**, 988–991.

Du,Z., Lang,S.M., Sasseville,V.G., Lackner,A.A., Ilyinskii,P.O., Daniel,M.D., Jung,J.U. and Desrosiers,R.C. (1995) Identification of a *nef* allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell*, **82**, 665–674.

Garcia,J.V. and Miller,A.D. (1991) Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. *Nature*, **350**, 508–511.

Gluzman,Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*, **23**, 175–182.

Graziani,A., Galimi,F., Medico,E., Cottone,E., Gramaglia,D., Boccaccio,C. and Comoglio,P.M. (1996) The HIV-1 Nef protein interferes with phosphatidylinositol 3-kinase activation. *J. Biol. Chem*., **271**, 6590–6593.

Grzesiek,S., Bax,A., Clore,G.M., Gronenborn,A.M., Hu,J.S., Kaufman,J., Palmer,I., Stahl,S.J. and Wingfield,P.T. (1996) The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase. *Nature Struct. Biol*., **3**, 340–345.

Haughn,L., Gratton,S., Caron,L., Sekaly,R.P., Veillette,A. and Julius,M. (1992) Association of tyrosine kinase p56lck with CD4 inhibits the induction of growth through the alpha beta T-cell receptor. *Nature*, **358**, 328–331.

Ho,W.Y., Cooke,M.P., Goodnow,C.C. and Davis,M.M. (1994) Resting and anergic B cells are defective in CD28-dependent costimulation of naive CD4+ T cells. *J. Exp. Med*., **179**, 1539–1549.

Janeway,C. (1992) The T cell receptor as a multicomponent signaling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu. Rev. Immunol*., **10**, 645–674.

Kestler,H.W., Ringler,D.J., Mori,K., Panicali,D.L., Sehgal,P.K., Daniel,M.D. and Desrosiers,R.C. (1991) Importance of the *nef* gene for maintenance of high virus load and development of AIDS. *Cell*, **65**, 651–662.

Kirchhoff,F., Greenough,T.C., Brettler,D.B., Sullivan,J.L. and Desrosiers, R.C. (1995) Absence of intact *nef* sequences in a long-term survivor with nonprogressive HIV-1 infection. *New Engl. J. Med*., **332**, 228–232.

Lee,C.-H., Saksela,K., Mirza,U.A., Chait,B.T. and Kuryan,J. (1996) Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell*, **85**, 931–942.

Lindemann,D., Wilhelm,R., Renard,P., Althage,A., Zinkernagel,R. and Mous,J. (1994) Severe immunodeficiency associated with a human immunodeficiency virus 1 NEF/3′-long terminal repeat transgene. *J. Exp. Med*., **179**, 979–807.

Lowell,C.A., Soriano,P. and Varmus,H.E. (1994) Functional overlap in the *src* gene family: inactivation of *hck* and *fgr* impairs natural immunity. *Genes Dev.*, **8**, 387–398.

Luo,T. and Garcia,J.V. (1996) The association of Nef with cellular serine/threonine kinase and its enhancement of infectivity are viral isolate dependent. *J. Virol*., **70**, 6493–6496.

Luria,S., Chambers,I. and Berg,P. (1991) Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. *Proc. Natl Acad. Sci. USA*, **88**, 5326–5330.

Mariani,R. and Skowronski,J. (1993) CD4 down-regulation by *nef* alleles isolated from human immunodeficiency virus type I-infected individuals. *Proc. Natl Acad. Sci. USA*, **90**, 5549–5553.

Mariani,R., Kirchhoff,F., Greenough,T.C., Sullivan,J.L., Desrosiers,R.C. and Skowronski,J. (1996) High frequency of defective *nef* alleles in a long-term survivor with nonprogressive HIV-1 infection. *J. Virol*., **70**, 7752–7764.

Miller,M.D., Warmerdam,M.T., Gaston,I., Greene,W.C. and Feinberg, M.B. (1994) The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *J. Exp. Med*., **179**, 101–113.

Myers,G., Berzofsky,J.A., Rabson,A.B. and Smith,T.E. (1993) *Human Retroviruses and AIDS*. Los Alamos National Laboratory, Los Alamos, NM.

Nishizumi,H., Taniuchi,I., Yamanashi,Y., Kitamura,D., Ilic,D., Mori,S., Watanabe,T. and Yamamoto,T. (1995) Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity*, **3**, 549–560.

Nunn,M.F. and Marsh,J.W. (1996) Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *J. Virol*., **70**, 6157–6161.

Regier,D.A. and Desrosiers,R.C. (1990) The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses*, **6**, 1221–1231.

Rhee,S.S. and Marsh,J.V. (1994) Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J. Virol*., **68**, 5156–5163.

Saksela,K., Cheng,G. and Baltimore,D. (1995) Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J*., **14**, 484–491.

Salghetti,S., Mariani,R. and Skowronski,J. (1995) Human immunodeficiency virus type 1 Nef and $p56^{lck}$ protein tyrosine kinase interact with a common element in CD4 cytoplasmic tail. *Proc. Natl Acad. Sci. USA*, **92**, 349–353.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sawai,E.T., Baur,A., Struble,H., Peterlin,B.M., Levy,J.A., Cheng-Mayer,C. (1994) Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc. Natl Acad. Sci. USA*, **91**, 1539–1543.

Sawai,E.T., Baur,A.S., Peterlin,B.M., Levy,J.A. and Cheng-Mayer,C. (1995) A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity. *J. Biol. Chem*., **270**, 15307–15314.

Shugars,D.C., Smith,M.S., Glueck,D.H., Nantermet,P.V., Seillier-Moiseiwitsch,F. and Swanstrom,R. (1993) Analysis of human immunodeficiency virus type 1 *nef* gene sequences present *in vivo*. *J. Virol*., **67**, 4639–4650.

Skowronski,J. and Mariani,R. (1995) Transient assay for Nef-induced down-regulation of CD4 antigen expression on the cell surface. In Karn,J. (ed.), *HIV-1: A Practical Approach*. IRL Press, Oxford, UK, pp. 231–242.

Skowronski,J., Parks,D. and Mariani,R. (1993) Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. *EMBO J*., **12**, 703–713.

Spina,C.A., Kwoh,T.J., Chowers,M.Y., Guatelli,J.C. and Richman,D.D. (1994) The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *J. Exp. Med*., **179**, 115–123.

Tanaka,M. and Herr,W. (1990) Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. *Cell*, **60**, 375–386.

Weiss,A. and Littman,D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263–274.

Wilson,A.C., Peterson,M.G. and Herr,W. (1995) The HCF repeat is an unusual proteolytic cleavage signal. *Genes Dev.*, **9**, 2445–2458.

Yokoyama,W.M., Koning,F., Kehn,P.J., Pereira,G.M.B., Stingl,G., Coligan,J.E. and Shevach,E.M. (1988) Characterization of a cell surface-expressed disulfide-linker dimer involved in murine T cell activation. *J. Immunol*., **141**, 369–376.

Zenner,G., Dirk zur Hausen,J., Burn,P. and Mustelin,T. (1995) Towards unraveling the complexity of T cell signal transduction. *BioEssays*, **17**, 967–975.

Zhu,L., van den Heuvel,S., Helin,K., Fattaey,A., Ewen,M., Livingstone,D., Dyson,N. and Harlow,E. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.*, **262**, 2050–2054.

Received on September 20, 1996; revised on November 4, 1996