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Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) ζ chain leads to TCR down-modulation

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The Nef protein of simian immunodeficiency virus (SIV) is dispensable for replication in established T-cell lines but essential for high level virus replication in the adult host, though the mechanism by which Nef contributes to this has remained unclear. We demonstrate here that SIV Nef binds to the ζ chain of the T-cell receptor (TCR). SIV Nef proteins that interact with TCR ζ in a yeast two-hybrid system also reduce T-cell surface expression levels of TCR αβ, CD3 and CD4. These findings are the first demonstration that Nef can bind directly to a component of the TCR–CD3 complex and modulate its surface expression.

Introduction

Despite the requirement for Nef for the maintenance of high virus loads and disease induction during simian immunodeficiency virus (SIV) infection of adult macaques (Kestler *et al.*, 1991), the exact mechanism(s) by which Nef exerts its function remains unclear. Nef is conserved among the primate lentiviruses, SIV and human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2, respectively). This 27–34 kDa protein is *N*-myristoylated (Allan *et al.*, 1985) and thus targeted to the inner surface of the plasma membrane, has no identified enzymatic activity and is incorporated into virus particles (Welker *et al.*, 1996). Multiple activities have been attributed to Nef including down-modulation of cell surface CD4 (Guy *et al.*, 1987; Benson *et al.*, 1993) and major histocompatibility antigen class I (MHC I) (Schwartz *et al.*, 1996; Collins *et al.*, 1998), enhancement of virion infectivity (Spina *et al.*, 1994; Chowers *et al.*, 1994) and enhancement or inhibition of T-cell activation (Baur *et al.*, 1994). Many of the activities attributed to Nef could affect the immune effector functions of productively infected cells, but it is not clear which activity is most relevant to disease induction. The one outcome of Nef expression, however, that continues to be demonstrated is altered T-cell signalling. Recently, Alexander *et al.* (1997) demonstrated that naturally occurring SIV and HIV-1 Nefs can overcome the requirement for IL-2 of an IL-2-dependent lymphoid cell line. In addition, the product of an extremely pathogenic SIV *nef* allele, SIVmac239YE (referred to as YE) Nef, generation of which was based on Nef amino acid sequence differences between the acutely pathogenic SIVsmmPBj (Fultz, 1991; Fultz & Zack, 1994) and prototypic SIVmac239 (Kestler *et al.*, 1990; referred to as 239), was demonstrated to interact directly with the ZAP70 protein kinase required for signalling through the T-cell receptor (TCR) (Luo & Peterlin, 1997). The observed effects of Nef on cell signalling are diverse, however, and cover a spectrum from direct T-cell hyperactivation, as with YE (Sasseville *et al.*, 1996), through inhibition of induction of early T-cell activation markers such as IL-2 (Luria *et al.*, 1991), Lck phosphorylation (Greenway *et al.*, 1995) and CD69 induction (Iafrate *et al.*, 1997), to exerting no effect (Page *et al.*, 1997).

Regardless of these differences in the possible mechanism(s) of Nef function, the absence of any enzymatic activity indicates that it exerts its function via protein–protein interactions. Many cellular proteins have been shown to bind directly to Nef *in vitro* and in transfected or infected cells. Interactions with cellular proteins involved in the immune function of T lymphocytes and monocytes, as well as cell signalling, have been well demonstrated in such assays as the yeast two-hybrid system (Rossi *et al.*, 1997), co-immunoprecipitation with Nef antisera (Harris & Coates, 1993) and ELISA (Greenway *et al.*, 1996). Among the proteins that have been shown to associate with Nef are Hck (Saksela *et al.*, 1995), Lck (Collete *et al.*, 1996; Greenway *et al.*, 1996), mitogen-activated protein kinase (MAPK; Greenway *et al.*, 1996), a serine/threonine kinase

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immunologically related to p21-associated kinase (PAK; Sawai *et al.*, 1994, 1995), β-COP (Benichou *et al.*, 1994), a CD4-associated thioesterase (Liu *et al.*, 1997; Watanabe *et al.*, 1997), proteasome subunit HsN3 (Rossi *et al.*, 1997), the μ clathrin-associated adaptor protein (Piguet *et al.*, 1998; Le Gall *et al.*, 1998), a vacuolar ATPase (Lu *et al.*, 1998) and CD4 (Rossi *et al.*, 1996). How this approximately 200-amino-acid protein accomplishes such varied functions is not clear, though it has been proposed to serve as a connector protein in protein trafficking pathways (Mangasarian *et al.*, 1997).

In order to understand SIV Nef function better we have characterized the functional and biochemical properties of the Nef proteins of the highly related molecular clones SIVmacJ5 and SIVmacC8 (Rud *et al.*, 1994; referred to here as J5 and C8, respectively). J5 is pathogenic whereas C8 is non-pathogenic, and pre-infection with C8 protects animals from subsequent challenge with either cell-free or cell-associated J5 (Almond *et al.*, 1995; Cranage *et al.*, 1997) in the same way that 239 with a deletion in *nef* protects from pathogenic challenge (Daniel *et al.*, 1992). These viruses differ only in their *nef* genes and 3′ long terminal repeats (LTR), resulting in a 4-amino-acid deletion and 2 amino acid substitutions in C8 Nef relative to J5 Nef. Using yeast two-hybrid technology, we found that the J5 Nef protein binds directly to the cytoplasmic domain of the TCR ζ chain, and that this interaction in T cells led to the down-modulation of the TCR–CD3 complex from cell surfaces.

Methods

■ **Generation and analysis of yeast strains.** All manipulations of the Y190 strain of *Saccharomyces cerevisiae* (Harper *et al.*, 1993) followed standard yeast genetic methods (Johnston, 1994). Recombinant yeast stably expressing Nef–Gal4 DNA binding domain (BD) fusion proteins were generated by targeted integration of *Xba*I-linearized Gal4 BD vectors into the *trp1* locus of strain Y190 as described (Fuller *et al.*, 1998). The BD fusion protein expression vector was pYTH9 (Fuller *et al.*, 1998).

Total yeast cellular extracts used for Western blot analyses were prepared as described previously (Johnston, 1994) by glass bead disruption and trichloroacetic acid precipitation. Cellular extracts were analysed by SDS–PAGE and immunoblotting with an antibody directed to the haemagglutinin (HA) tag present in the BD and Gal4 activation domain (AD) fusion proteins immediately C-terminal to the BD or AD portion (12CA5, Boehringer Mannheim), followed by incubation with a goat anti-mouse–peroxidase conjugate and chemiluminescent detection (ECL, Amersham).

■ **cDNA library and yeast two-hybrid assay.** The cDNA expression library in pACT2, kindly provided by M. Suda (Glaxo Nippon, Japan), has been described (Fuller *et al.*, 1998), and was generated from pooled non-activated and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) activated H9 T cells. Screening of the cDNA library in the Y190 strain of *S. cerevisiae* expressing the SIV J5 Nef–BD was performed as described by Fuller *et al.* (1998), with $1{\cdot}2 \times 10^7$ transformants screened. Transformants were grown on minimal media agar plates supplemented with the appropriate amino acids but lacking histidine, tryptophan and leucine, and also containing a competitive inhibitor of the *HIS3* gene product, 3-amino-1,2,4-triazole (25 mM). The selected transformants were stained for β-galactosidase using a colony lift, freeze–thaw fracture technique (Fuller *et al.*, 1998), and colonies containing β-galactosidase were restreaked twice to generate clonal strains which were then stained again for β-galactosidase and lysed for back-extraction of cDNA-containing pACT2 plasmids. Plasmids amplified in *E. coli* (DH5α) were analysed for the presence and size of cDNA inserts by PCR with pACT2-specific primers Gal4ADF (5′ ATGGATGATGTATATAACTATCTATTCG 3′) and ACT2MCSR (5′ GAAAGAAATTGAGATGGTGCACG 3′). Quantitative solution assays for β-galactosidase activity were performed as previously described (Harshman *et al.*, 1988).

■ **PCR and subcloning.** The J5 and C8 *nef* genes were amplified by PCR with *Pfu* polymerase (Stratagene) according to the manufacturer's recommendations, with primers cc1203 (5′ CCGGAATTCCATGGGTGGAGCTATTTCC 3′) and cc951 (5′ CCGGAATTCTCAGCGAGTTTCCTTCTTGTC 3′), and M13RF DNA *nef* templates (kindly provided by N. Almond, NIBSC, UK). PCR products were digested with *Eco*RI, subcloned into the yeast BD expression vector pYTH9 (Fuller *et al.*, 1998), and DNA was sequenced according to established protocols for ABI automated sequencing (Perkin Elmer). The sequenced *Eco*RI inserts were then subcloned into the mammalian expression vector pSA90 (previously named pGW1HG; Chapman *et al.*, 1991). The *nef* gene of 239 was similarly amplified with primers cc1203 and cc951 with p239SpSp3′ as template. To repair the premature stop codon in 239 *nef*, overlapping PCR was performed using primer pairs cc1203 plus mac239-2 (5′ CAAGTCATCATCTTCCTCATCTATATCATC 3′) and mac239-1 (5′ GATGAGGAAGATGATGACTTGGT 3′) plus cc951 in separate 50 μl PCRs, and 4 μl of each of these products was then added to a third PCR with the outer primers cc1203/cc951 to regenerate the full-length *nef* gene. The same strategy was used to introduce mutations into the J5 *nef* gene. For mutation of the proline-rich motif (PXXP at amino acid positions 104–107 in J5 Nef) to AXXA, first-round PCRs were performed with primers cc1203 plus SIVPXXP-2 (5′ TAGGGCAACTCGTGCCATCACTGGTACCCCTAC 3′) and SIVPXXP-1 (5′ ATGGCACGAGTTGCCCTAAGAACAATGAGT 3′) plus cc951. For mutation of the RR motif (amino acids 108–109) to LL, first-round PCRs were performed with primer cc1203 plus SIVRRLL-2 (5′ TCTATGTTTTTTTGCACTGTAATAAATCC 3′) and SIVRRLL-1 (5′ AGTGCAAAAAAACATAGAATCTTAGACAT 3′) plus cc951. Lastly, to mutate the DMYL insertion in J5 Nef (positions 143–146) primer SIVDMYL-2 (5′ TTCTGCG[T/A]ACATGT[C/T]TAAGATTCTATGTCT 3′) was used with cc1203 and primer SIVDMYL-1 (5′ TTA[G/A]ACATGT[A/T]-CGCAGAAAAGGAGGAAGGC 3′) was used with cc951 in first-round PCRs. To generate Nef–GFP (green fluorescent protein) expression vectors, the J5 and C8 *nef* genes were amplified by PCR with primers cc1203 and gfp1 (5′ GTGGATCCCGGCGAGTTTCCTTCTTGTC 3′). SIV Nef–GFP PCR products were digested with *Bam*HI and *Eco*RI and ligated to *Bam*HI/*Eco*RI-digested pEGFP-N1 (Clontech).

The Src homology domain 3 (SH3 domain) of Hck was amplified by RT–PCR with primers Hck1CA (5′ TATGGCCATGGTCATCGTGGTTGCCCTGTATGAT 3′) and Hck2CA (5′ CGAATTCAATGTCAACGCGGGCGACATAGTTGCT 3′) after reverse transcription of total mRNA from the promonocytic U937 cell line with Moloney murine leukaemia virus RT and oligo(dT) as primer. Products were digested with *Eco*RI and *Nco*I and subcloned into identically digested pACT2.

The AP50 open reading frame was amplified by PCR with *Taq* polymerase (Promega) according to the manufacturer's recommendations, with primers AP501 (5′ CCGCTCGAGCCCGGGGATGATTGGAGGCTTATTCAT 3′) and AP502 (5′ CCGCTCGAGCTAGCAGCGAGTTTCATAAAT 3′) using clone no. 44395 of the I.M.A.G.E. consortium cDNA library (Lennon *et al.*, 1996) as template. PCR products were digested with *Sma*I and *Xho*I and were ligated to identically prepared pACT2.

■ **Cells and transfections.** The $CD4^+$ Jurkat T-cell line JJK and the $CD4^-$ Jurkat T-cell line J6 were grown in RPMI-1640 (Sigma) supplemented with 10% foetal calf serum (FCS; Gibco) and the 293T fibroblast cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% FCS. All culture media were supplemented with 20 mM L-glutamine, and all cell lines were maintained at 37 °C in 5% CO_2.

JJK and J6 cells (10^7) were electroporated with a Gene Pulser II apparatus (300 V, 950 μF; Bio-Rad) at room temperature in 0·4 cm cuvettes, with 15 μg plasmid in 500 μl RPMI supplemented with 20% FCS. The cells were resuspended following electroporation in 10 ml complete RPMI. 293T cells at 50% confluency were transfected by a phospholipid-based method with 5 μg plasmid in 10 μl Transfectam (Gibco) per well in a 24-well plate in 200 μl serum-free RPMI for 2 h at 37 °C. Cells were rinsed twice with DMEM/FCS and re-fed with the same medium.

Expression of SIV Nef was assessed by Western blot analysis using monoclonal antibody KK7, directed at amino acids 61–80 of J5 and C8 Nefs (kindly provided by K. Kent, NIBSC).

■ **Flow cytometry.** For analysis of cell surface antigen expression, flow cytometry was performed 48 h post-transfection. Cells (10^5) were washed with PBS, resuspended in 200 μl PBS containing 2% BSA, 0·2% sodium azide and saturating quantities of conjugated primary antibody, and incubated on ice for 30 min. Cells were then washed with PBS and resuspended in 0·5 ml PBS containing 0·2% sodium azide, and analysed with an Epics XL flow cytometer (Coulter). Viable cells were selectively gated based on forward and side scatter profiles with each analysis representing 10^4 events. Data analysis was performed with the WinMDI software package (J. Trotter, The Scripps Research Institute). The following antibodies were used: anti-CD3 (UCHT-1; Coulter), anti-CD4 (Q4120; Sigma), anti-CD71 (DF 1513; Sigma), anti-TCR αβ (WT 31; Beckton Dickinson) and anti-CD69 (31954X; Pharmingen).

■ **TCR ζ immunoprecipitations.** TCR ζ was immunoprecipitated from JJK cells following transient transfection of 10^7 cells with Nef–GFP fusion protein expression plasmids. Cells (10^7) were lysed 48 h post-transfection in immunoprecipitation buffer (1 M Tris–HCl, pH 8·0; 1% octyl β-D-thioglucopyranoside; 2 M NaCl; 1 M sodium orthovanadate; 0·076 U/ml aprotinin; 10 μg/ml leupeptin; 10 μg/ml pepstatin A; 10 μg/ml chymostatin; 800 μM PMSF; 0·2 mM EDTA; 1 mM iodoacetamide). Lysates were precleared with 50 μl of a 40% slurry of agarose–protein A, and then incubated with 5 μl rabbit polyclonal anti-TCR ζ antiserum (kindly provided by J. Tite, Glaxo Wellcome) and 50 μl 40% agarose–protein A for 2 h at 4 °C. Immunoprecipitates were washed six times with immunoprecipitation buffer, resuspended in 50 μl SDS–PAGE loading buffer, and 15 μl was loaded onto a 12% SDS–acrylamide gel without boiling the sample. This was necessary in order to maintain the fluorescent capability of the GFP and Nef–GFP proteins. Total cellular lysates were prepared by lysing 10^5 PBS-washed cells in 100 μl SDS–PAGE loading buffer and 15 μl was loaded onto a 12% SDS–acrylamide gel. Gels were scanned with a Fluorimager SI (Vistra Fluorescence) to detect fluorescing proteins. Captured images were analysed using the ImagequaNT software package (Molecular Devices).

Results

Interaction of J5 Nef with the TCR ζ chain in the yeast two-hybrid system

As an initial step towards characterizing the J5 and C8 Nef proteins and identifying differences that might explain their drastically different effects on the pathogenic outcome of infection *in vivo*, we screened an H9 T cell cDNA expression library to identify cellular proteins that bound to J5 Nef using yeast two-hybrid technology in *S. cerevisiae*. The bait, J5 Nef, was expressed as a Gal4 BD fusion protein via targeted integration into the Y190 strain of *S. cerevisiae*. The cDNAs in the library were expressed from the episomal vector pACT2 as Gal4 AD fusion proteins. Successful Nef–protein interactions were identified by the growth of yeast transformants on agar lacking histidine and subsequent staining for β-galactosidase. From the H9 T cell cDNA library we obtained three clones expressing the cytoplasmic domain of the invariant ζ chain of the TCR (Fig. 1*a*). Clones 5.1 and 2.21 were identical and expressed the C-terminal 56 amino acids of TCR ζ, whereas clone 1.11 expressed the entire cytoplasmic domain (amino acids 53–163; Fig. 1*a*). Clone 1.11 also contained two amino acid substitutions (E60D, P61A) and one amino acid insertion (Q between amino acids 100 and 101) relative to the previously published human TCR ζ sequence (Weissman *et al.*, 1988). This is the first report of Nef binding directly to a component of the TCR–CD3 complex. The requirement for both J5 Nef–BD and TCR ζ–AD to obtain a positive signal in this yeast two-hybrid assay is demonstrated in Fig. 1(*b*), where omission of either of the binding partners, or inclusion of the BD or AD alone, prevented growth on histidine-deficient agar and the production of β-galactosidase.

The abilities of other SIV Nef–BD fusion proteins to interact with the cytoplasmic domain of the TCR ζ–AD fusion proteins were also examined in *S. cerevisiae* using both filter-based and quantitative solution assays for β-galactosidase. In addition to J5 Nef, the prototypic 239 Nef protein interacted with the cytoplasmic domain of TCR ζ (Table 1). In contrast, the attenuated C8 Nef protein did not bind TCR ζ, resulting in β-galactosidase activities three orders of magnitude lower than those of the pathogenesis-conferring J5 and 239 Nef proteins. Mutation of the PXXP and RR motifs in the central conserved region of J5 Nef, which are involved in Nef binding to SH3 domains in protein tyrosine kinases (PTKs) (Saksela *et al.*, 1995), binding to cellular serine/threonine kinases (Sawai *et al.*, 1995) and increasing infectivity of virus stocks (Saksela *et al.*, 1995), had only a minimal or no effect on J5 Nef binding to TCR ζ (Table 1). Similarly, mutation of the 4-amino-acid insertion in J5 relative to C8 (DMYL to NMYA, positions 143–146) did not prevent interaction of J5 Nef with TCR ζ, and actually led to this mutant exhibiting the greatest levels of β-galactosidase activity in all interactions with AD fusion proteins (Table 1). The N-terminal 92 amino acids of the 239 Nef, expressed by the parental gene containing a premature stop codon, were unable to bind to the TCR ζ cytoplasmic domain, suggesting that the domain(s) either for binding to TCR ζ or for appropriate conformational presentation of the binding domain are not present solely in the 92 N-terminal amino acids. Unexpectedly, none of the SIV Nef–BD fusion proteins interacted with the SH3 domain of Hck PTK (HcK-SH3) fused to Gal4 AD (Table 1), although interaction between

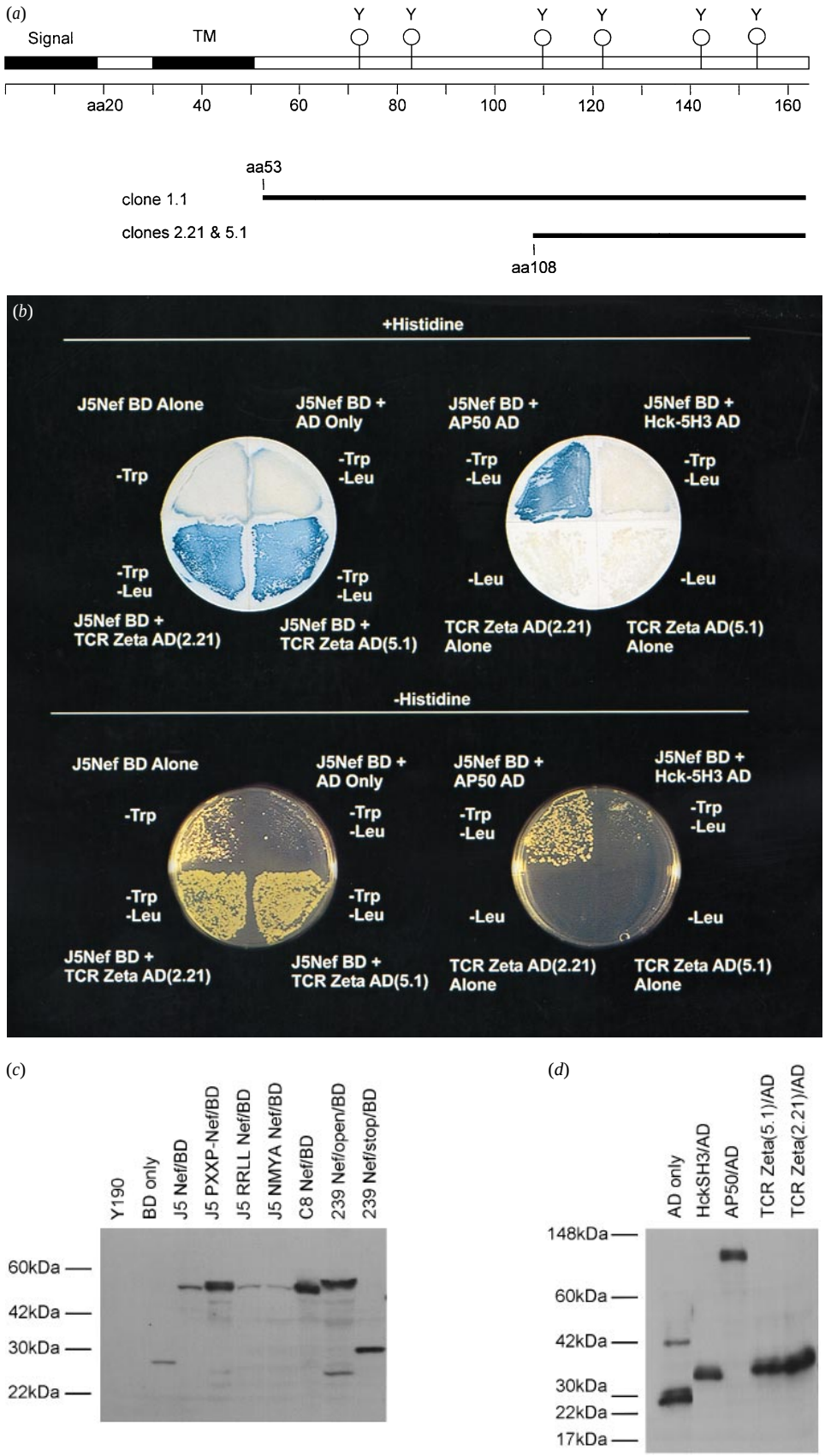


Fig. 1. For legend see facing page.

Table 1. Quantitative analysis of interactions between SIV Nef and cellular proteins in a yeast two-hybrid system

Interactions between AD and BD fusion proteins in *S. cerevisiae* were quantified using a solution assay for *β*-galactosidase in yeast cellular extracts (Harshman *et al.*, 1988). Numerical values represent arbitrary *β*-galactosidase units normalized for the amount of input biomass determined by the absorbance at 600 nm. ND, Not determined.

	AD fusion						
Nef–BD fusion	ζ/1.11	ζ/5.1	ζ/2.21	Hck-SH3	AP50	AD only	None
J5	365·6	224·8	195·4	< 0·1	44·2	< 0·1	0·2
C8	< 0·1	0·1	0·2	< 0·1	7·9	< 0·1	< 0·1
J5/PXXP⁻	305·8	203·9	116·0	< 0·1	69·9	< 0·1	< 0·1
J5/RRLL	233·3	50·6	ND	0·2	88·1	0·1	0·1
J5/NMYA	457·0	352·9	339·4	0·2	118·4	0·1	0·1
239	312·1	126·7	208·9	< 0·1	59·8	< 0·1	< 0·1
239/stop (1–92)	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
BD only	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
None	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1

HIV-1 Nef and Hck-SH3 is one of the strongest interactions identified thus far for a PXXP motif (Saksela *et al.*, 1995). Interaction of SIV Nef with the cellular protein AP50 served as a positive control in our assay. AP50 is the medium (μ) chain of the heterotetrameric clathrin coat-associated adaptor protein complex AP2 (reviewed in Robinson, 1994), which is involved in the endocytosis of transmembrane proteins in clathrin-coated pits, and has been demonstrated recently to interact with 239 Nef (Piguet *et al.*, 1998; Le Gall *et al.*, 1998). Interestingly, the AP50–AD fusion protein was the only one with which the C8 Nef–BD fusion protein demonstrated any appreciable interaction (Table 1).

The expression of Nef–BD and cellular protein–AD fusion proteins was confirmed by Western blot analysis of yeast cellular lysates with an antibody specific for an HA tag present just C-terminal to the Gal4 domains (Fig. 1*c*, *d*). The proteins detected were all of the expected sizes except those that were fused to the Gal4 AD, which were all approximately 3–5 kDa larger than expected, possibly due to translational readthrough to a downstream stop codon. Gal4 BD expressed alone was larger than expected (Fig. 1*c*), because there are no stop codons in the multiple cloning site and a fortuitous stop codon was utilized further into the vector. The levels of expression of the Nef–BD fusion proteins were variable, but did not correlate with the strength of the *β*-galactosidase signals obtained from yeast expressing these proteins (Table 1).

Co-immunoprecipitation of TCR ζ and Nef

To determine whether SIV Nef also interacted with TCR ζ in T cells, we generated plasmids expressing the J5 and C8 Nef proteins fused to the N terminus of GFP and transiently transfected these plasmids into the JJK T-cell line, in which Nef has been shown previously to down-modulate cell surface CD4

Fig. 1. Interaction of SIV J5 Nef with the cytoplasmic domain of TCR ζ demonstrated in the yeast two-hybrid system. (*a*) A schematic representation of the TCR ζ chain with tyrosines (Y) indicated in the immunoreceptor tyrosine-based activation motifs (ITAMs). Regions of TCR ζ contained in cDNA clones identified by their ability to bind to J5 Nef–BD in the yeast two-hybrid system are indicated by black lines. TM, trans-membrane domain. (*b*) Interactions between the J5 Nef–BD and TCR ζ–AD clones 5.1 and 2.21 are shown. The TCR ζ–AD clones were obtained by screening a cDNA–AD expression library generated from pooled unstimulated and stimulated H9 cells by yeast two-hybrid technology with J5 Nef–BD as the bait. Successful protein–protein interactions allowed expression of *β*-galactosidase from the Gal4 promoter, and therefore conversion of the substrate (X-Gal) to a blue product in a filter lift, freeze–fracture technique (labelled +Histidine, as the yeast were grown in the presence of histidine). Successful protein–protein interactions also allowed growth in the absence of histidine due to increased transcription of the *HIS3* gene, again from the Gal4 promoter (−Histidine). Media lacking tryptophan provided selective pressure for maintenance of the BD expression cassette, whereas media lacking leucine provided selective pressure for maintenance of the AD expression cassettes. Images of individual discs and plates shown were digitally assembled after image capture because the yeast in different quadrants of each plate had different auxotrophic requirements. (*c*, *d*) Expression of Nef–BD (*c*) and cellular protein–AD (*d*) fusion proteins in *S. cerevisiae* strain Y190 was examined by Western blot analysis with an anti-HA monoclonal antibody. There is an HA tag immediately C-terminal of both the BD and AD portions of the fusion proteins. Data shown were derived from single yeast colonies, though at least two colonies were analysed for each expression vector and resulted in similar expression levels and protein banding patterns.

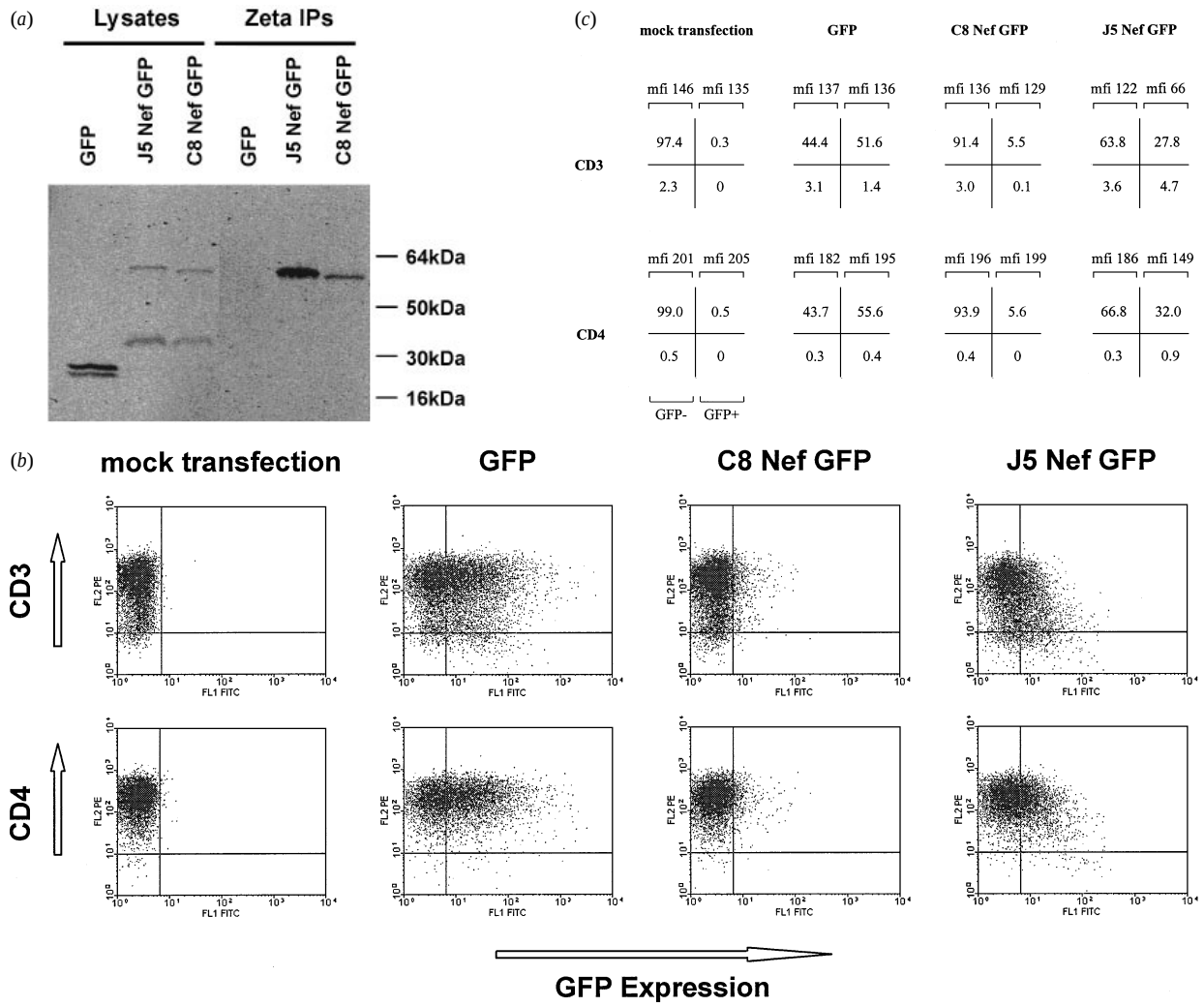
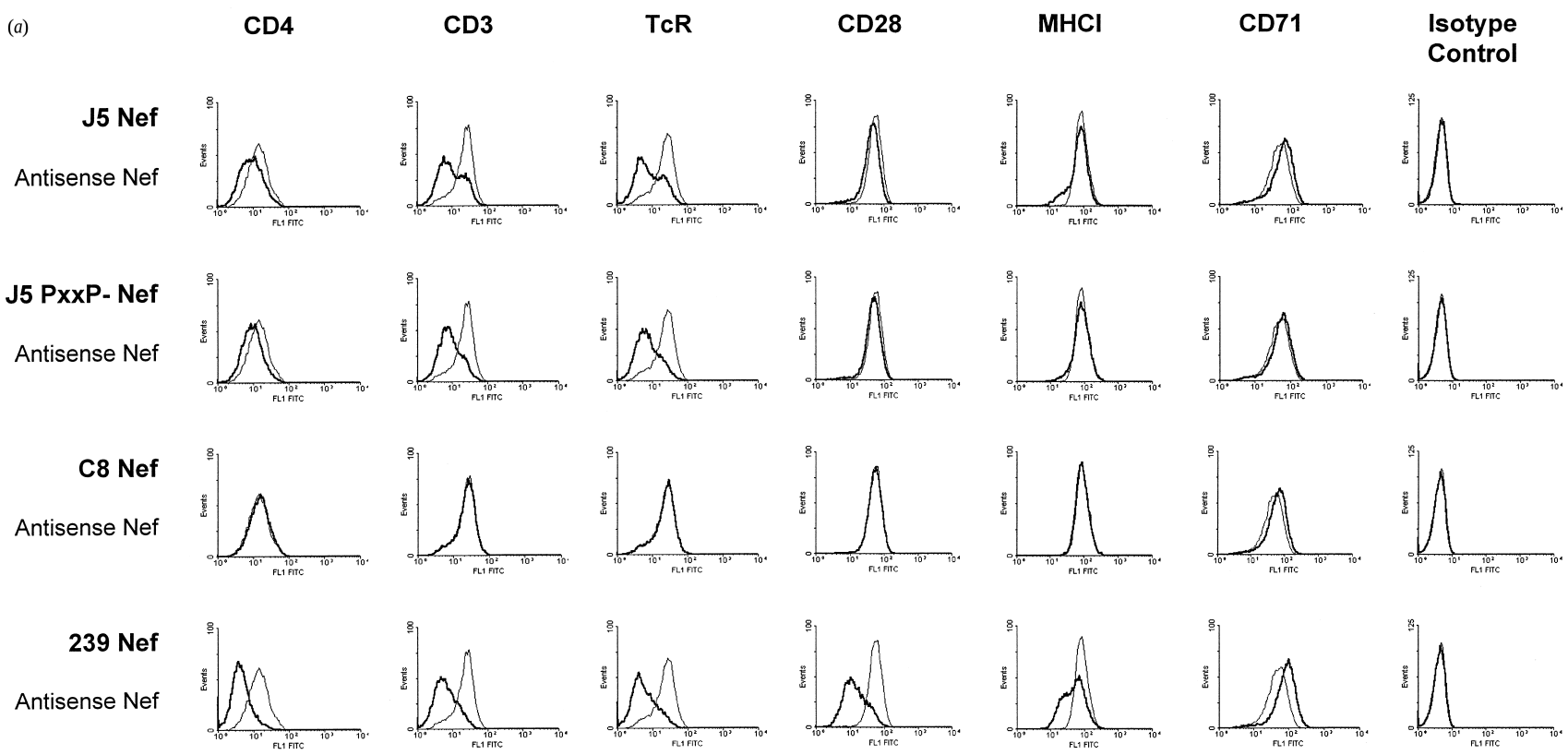


Fig. 2. Co-immunoprecipitation of SIV Nef–GFP fusion protein with TCR ζ. (*a*) JJK cells were transfected with plasmids expressing either GFP or the indicated Nef–GFP fusion protein, and total cellular lysates were harvested 48 h later. Cells ($7{\cdot}5 \times 10^4$ cell equivalents) were loaded directly in the three left-hand lanes. In parallel, 3×10^6 cell equivalents were immunoprecipitated with a rabbit polyclonal antiserum to TCR ζ and loaded in the three right-hand lanes. GFP and Nef–GFP fusion proteins were visualized using a scanning fluorimeter and appear as dark bands. (*b*) Plasmids expressing the indicated GFP protein were transfected into JJK cells and the cells were analysed 48 h later by two-colour flow cytometry for surface CD3 or CD4 (phycoerythrin, PE) and green fluorescence (GFP). (*c*) The percentages of cells in each of the four quadrants of the dot plots in (*b*) are shown, as are the mean PE fluorescence intensities in the GFP^- and GFP^+ populations.

and to inhibit CD3-mediated T-cell activation (Iafrate *et al.*, 1997). Immunoprecipitation of TCR ζ and detection of the Nef–GFP fusions by SDS–PAGE and direct fluorimetric analysis of the gel indicated that these Nef proteins associated with TCR ζ (Fig. 2*a*). However, the efficiencies with which the Nef proteins were co-immunoprecipitated by TCR ζ differed, with the J5 Nef–GFP fusion protein exhibiting the highest efficiency, as evidenced by comparison of the intensity of the Nef–GFP bands in total cellular lysates versus TCR ζ co-immunoprecipitates (Fig. 2*a*). Although expressed to a high level, GFP was not detected in TCR ζ immunoprecipitates (Fig. 2*a*), indicating that the GFP component of the Nef–GFP fusion proteins did not contribute to their interactions with TCR ζ. The identity of the extra band in the total cellular lysates containing Nef–GFP fusion proteins is not clear, but it might result from translational initiation at an internal methionine 89 amino acids from the C terminus of the J5 and C8 Nef proteins.

In order to demonstrate the functional capabilities of these Nef–GFP fusion proteins, we examined their abilities to modulate the levels of T-cell surface antigens, such as CD4, using two-colour flow cytometry (Fig. 2*b*, *c*). As expected, the J5 Nef–GFP fusion protein reduced cell surface CD4 levels, with the lowest levels of CD4 present on cells exhibiting the brightest green fluorescence. Interestingly, the cell surface levels of CD3 were also greatly reduced by expression of the J5 Nef–GFP fusion protein. Transient expression of the C8



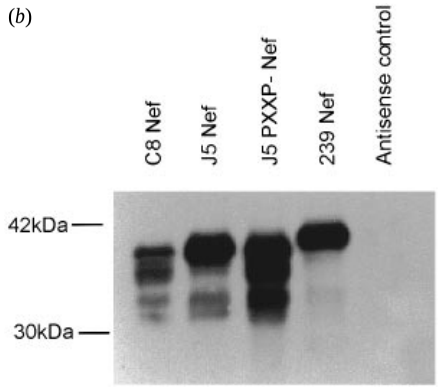


Fig. 3. Modulation of expression levels of T-cell surface proteins by SIV Nef. (*a*) JJK cells were electroporated with plasmids expressing C8, J5, J5 PXXP^{-} or 239 Nef proteins or an antisense control. Cells were stained 48 h later for the indicated surface antigen or were stained with an isotype control antibody, and then analysed by flow cytometry for the intensity of antibody staining. Thin lines represent the surface staining profiles of cells transfected with antisense controls, whereas thick lines represent the staining profiles of cells expressing the indicated Nef proteins. Horizontal axis, increasing cell surface staining; vertical axis, increasing cell number. (*b*) Nef expression in 293T cells was examined by Western blot analysis 48 h after transfection, with an SIV Nef-specific monoclonal antibody (KK7).

Table 2. Quantitative analysis of T-cell surface protein modulation by SIV Nef

Cells were transiently transfected with plasmids expressing the indicated Nef protein and stained for surface antigens 48 h later as described in Methods. Cells were also stained with an isotype control murine monoclonal antibody, and a window was set to the right of this peak that excluded 99% of these cells. The values shown represent the percentage of cells that were antigen-positive and for which the levels of fluorescent staining were above the isotype control levels. These values represent the means of three independent experiments; SD shown in parentheses. ND, Not determined.

	Surface antigen					
Nef Protein	CD3	CD4	TCR $\alpha\beta$	CD71	CD28	MHC I
JJK T-cell line						
J5	52·6 (8·9)	53·2 (11·4)	49·6 (7·4)	96·5 (0·7)	98·0 (0·7)	98·9 (0·7)
C8	90·6 (1·5)	85·6 (5·7)	88·6 (1·7)	97·8 (0·4)	99·2 (0·4)	99·7 (0·2)
J5/PXXP$^-$	50·1 (10·6)	59·4 (13·2)	43·1 (12·1)	96·9 (0·9)	98·4 (0·7)	99·4 (0·4)
239	49·2 (13·4)	32·9 (15·7)	46·7 (13·9)	97·7 (0·2)	82·8 (10·9)	99·3 (0·3)
Antisense control	83·8 (7·9)	81·3 (6·2)	87·0 (2·3)	96·8 (0·9)	98·9 (0·2)	99·7 (0·1)
J6 T-cell line						
J5	41·0 (9·4)	1·9 (0·2)	ND	87·8 (1·0)	94·5 (2·2)	98·6 (0·5)
C8	86·1 (1·9)	5·1 (2·2)	ND	78·6 (3·6)	96·9 (1·3)	99·6 (0·2)
J5/PXXP$^-$	37·8 (10·7)	1·5 (0·2)	ND	84·5 (2·2)	93·9 (1·8)	98·9 (0·2)
239	41·0 (6·4)	1·5 (0·2)	ND	91·0 (2·7)	63·1 (6·9)	97·7 (0·3)
Antisense control	78·7 (4·4)	3·6 (0·9)	ND	76·3 (6·8)	96·9 (1·6)	99·6 (0·3)

Nef–GFP fusion protein invariably failed to yield cells with high levels of green fluorescence (Fig. 2*b*), though in the few cells that did exhibit some fluorescence there did not appear to be any down-modulation of CD3 or CD4 surface expression levels.

J5 Nef down-modulates CD4 and the TCR–CD3 complex from JJK T-cell surfaces

The differences observed between the J5 and C8 Nefs in their ability to bind to TCR ζ in the yeast two-hybrid and T-cell co-immunoprecipitation assays prompted us to examine whether there were differences in the consequences of these interactions in T cells. We transiently transfected SIV Nef expression plasmids into JJK T cells and used flow cytometry to quantitate surface antigen levels of CD4, CD3, TCR $\alpha\beta$, CD28 and MHC I. Consistent with the abilities of the J5 and 239 Nef proteins to bind to TCR ζ in the yeast two-hybrid system, they both down-modulated cell surface CD3 and TCR $\alpha\beta$, as well as CD4 (Fig. 3*a* and Table 2). These data demonstrate for the first time that SIV Nef decreases the surface expression of the antigen-specific TCR complex. In contrast to their potent abilities to down-modulate the TCR–CD3 complex, the J5 and 239 Nef proteins only slightly down-modulated MHC I, and only the 239 Nef protein decreased cell surface CD28 levels. Importantly, the attenuated C8 Nef protein did not alter surface levels of CD3, CD4 or any antigen examined (Fig. 3*a*). Reduction in the amount of TCR–CD3 complex on the cell surface was not dependent on association with and down-modulation of CD4, since J5 Nef was able to down-modulate cell surface CD3ε levels on the J6 T-cell line (Table 2). Cell surface levels of the transferrin receptor (CD71) were not significantly altered by expression of any of the Nef proteins (Fig. 3*a*), indicating that Nef did not alter protein trafficking universally to and from the cell surface.

We included within each set of transfections the plasmid pEGFP-N1, which routinely provided transfection efficiencies between 60–80% as determined by flow cytometry (data not shown). The reproducibility of our transient transfection assay is evident by the SD associated with mean percentages of surface antigen-positive cells (Table 2), which are on average 9% of their respective means. The numbers in Table 2 actually under-represent the extent of surface antigen down-modulation, due to the stringent criteria we used in setting the lower limits of the gate for antigen positivity based on the isotype control profile. For example, the 239 Nef protein reproducibly reduced cell surface MHC I levels as observed on the flow cytometric profile (Fig. 3*a*). This is not reflected in the data in Table 2 because the moderate reduction in fluorescence intensity does not shift the intensity below the upper limit of the fluorescence intensity obtained following staining with the isotype control (Fig. 3*a*). The abilities of the expression plasmids to direct Nef expression was examined by transfection of 293T cells and Western blot analysis of cell lysates with a monoclonal antibody directed against Nef (Fig. 3*b*). In general, all of the Nefs were of the expected size and were expressed to roughly equivalent levels except for C8 Nef, expression of which was routinely somewhat less than that of the other Nefs.

Discussion

Here we have examined the interaction of SIV Nef with T-cell proteins and identified the cytoplasmic tail of the invariant ζ chain of the TCR as a cellular partner of the functionally uncharacterized J5 Nef and the prototypic 239 Nef. The observation that the 239 Nef bound to TCR ζ and down-modulated the TCR–CD3 complex from JJK T cells indicates that the capacity to modify T-cell surface phenotype and function in such a potent manner is not an unusual property of J5 Nef. The ability of the SIVmac Nef proteins examined here to bind to TCR ζ and down-modulate the TCR–CD3 complex suggests that these functional properties might contribute to the pathogenic outcome of infection *in vivo*, by reducing the availability of the antigen-specific TCR–CD3 complex for stimulation by antigen on antigen-presenting cells. Down-modulation of the TCR–CD3 complex, which initiates the T-cell signalling cascade when bound by the appropriate complex of MHC and antigenic peptide (reviewed in Wange & Samelson, 1996), would be expected to have severe quantitative and qualitative effects on the ability of the T cell to respond to such stimulation, and reduce its ability to provide T cell help.

The inability of C8 Nef to perform any of the functions performed by J5 Nef, including CD4 down-regulation (Fig. 3*a*), could be due to inappropriate folding of the protein. Inherent instability of the C8 Nef protein is suggested by the somewhat lower levels of C8 Nef observed in 293T cells (Fig. 3*b*) and by the paucity of cells fluorescing brightly after transfection of plasmid expressing the C8 Nef–GFP fusion protein (Fig. 2*b*). The C8 Nef protein nevertheless seemed to be stabilized by fusion at its N terminus to the Gal4 BD (Fig. 1*c*). There is tremendous selective pressure to maintain the 4 amino acids deleted in C8 Nef relative to J5 Nef, as demonstrated by the repair of the deletion in C8 Nef *in vivo* with ensuing increases in virus load and disease progression (Whatmore *et al*., 1995). The single mutant we generated in this region of J5 Nef, replacing the DMYL insertion with NMYA, did not reduce the ability of J5 Nef to bind to TCR ζ (Table 1), suggesting that the specific amino acids in the first and fourth positions in this motif do not determine TCR ζ binding capability. It will require further analysis to determine whether the 2 central amino acids in this motif are similarly dispensable for this function. It is likely that this motif has a more general effect on protein structure. This is supported by the observation (Table 1) that the C8 Nef–BD fusion protein did not interact with AP50, despite the presence of tyrosines at positions 28 and 39 in Nef which were demonstrated by Piguet *et al*. (1998) to be required for interaction between 239 Nef and A50. In addition, mutation of the SH3-binding PXXP domain, which contributes to interactions between Nef and PTKs and PAKs (Saksela *et al*., 1995; Sawai *et al*., 1995), to the enhanced infectivity of SIV and HIV-1 virions (Saksela *et al*., 1995), and to MHC I down-modulation (Greenberg *et al*., 1998), did not prevent binding to TCR ζ. The failure of any of the SIV Nef–BD fusion proteins to interact with Hck-SH3–AD suggests that there might be differences in the repertoires of cellular binding partners that are important for SIV and HIV-1 Nef proteins to exert their functions.

Based on the regions of TCR ζ expressed by cDNA clones 1.11, 2.21 and 5.1, and the nature of the yeast two-hybrid system used for examining TCR ζ–Nef binding, the following conclusions can be drawn about the regions responsible for interaction with Nef. Firstly, the cytoplasmic domain of TCR ζ is sufficient for interaction with SIV Nef, and the membrane-proximal immunoreceptor tyrosine-based activation motif (ITAM) is not required, as clones 5.1 and 2.21 contained only the two distal ITAMs (Fig. 1*a*). These motifs are critical for appropriate interactions with Src homology domain 2- (SH2) containing proteins, such as the ZAP70 kinase, in the T-cell signalling pathway (reviewed in Wange & Samelson, 1996). It is possible that the products of different *nef* alleles will interact differently with TCR ζ, as seen with the J5 Nef/RRLL mutant (Table 1), which interacted more strongly with the TCR ζ clone containing all three ITAMs than with the clone containing only the two distal ITAMs. Secondly, phosphorylation of the tyrosines in the ITAMs is not required for interaction with Nef, since *S. cerevisiae* has not yet been shown to express PTKs so TCR ζ expressed in *S. cerevisiae* will therefore not contain phosphorylated ITAMs. Thirdly, *N*-myristoylation is not required for Nef to achieve a conformation appropriate for binding to TCR ζ, since the Nef–BD fusion protein used in this yeast two-hybrid assay was probably not myristoylated and was able to get to the nucleus and participate as a protein binding partner. However, in an infected mammalian cell this post-translational modification is probably necessary for targeting Nef to the plasma membrane, where the T-cell signalling machinery resides. Finally, there were no requirements for other TCR–CD3 components in order for Nef and TCR ζ to interact, nor were Src or Syk family PTKs required, either as bridging molecules or kinases.

It is conceivable, as has been proposed previously with regard to CD4 down-regulation (Mangasarian *et al*., 1997; Piaguet *et al*., 1998), that Nef bridges the cytoplasmic domains of T-cell surface proteins such as TCR ζ to components of the protein trafficking machinery. Consistent with this model, the SIV Nefs which were capable of binding to TCR ζ in the yeast two-hybrid system, and of reducing T-cell surface expression levels of TCR–CD3, were also capable of interacting with the AP50 (Table 1) and AP47 (data not shown) subunits of the clathrin coat-associated adaptor protein complexes AP2 and AP1, respectively, as demonstrated for 239 Nef by Piguet *et al*. (1998) and Le Gall *et al*. (1998). There are probably other factors that quantitatively and qualitatively influence the association of different SIV Nefs with TCR ζ. The detection of C8 Nef in TCR ζ immunoprecipitates, despite its inability to bind to TCR ζ in yeast, suggests that it nevertheless makes its way to the plasma membrane and associates at least weakly

with the TCR–CD3 complex. However, its association is quantitatively and qualitatively different, in that it does not down-modulate the TCR–CD3 complex from the T-cell surface.

The ability of SIV Nef to disrupt T-cell signalling has been extensively investigated, and thus far there is no consensus as to whether Nef enhances or inhibits T-cell activation, or has any effect at all. A recent report has demonstrated that the product of an extremely pathogenic SIV *nef* allele generated from 239 *nef*, YE Nef, which behaves identically to the SIVsmmPBj14 Nef also containing a tyrosine at position 17, binds directly to the ZAP70 PTK and enhances its kinase activity (Luo & Peterlin, 1997). This unique biochemical interaction is consistent with the high level of T-cell activation observed in SIVsmmPBj14- and YE-Nef-infected animals (Fultz & Zack, 1994; Sasseville *et al*., 1996) and the abilities of these viruses to replicate in unstimulated PBMCs (Fultz 1991; Du *et al*., 1995). These findings, along with those we have presented here, suggest that the extent to which Nef is intimately associated with the T-cell signalling machinery might contribute to the rate of disease progression. The strategy that might be used by the J5 and 239 Nef proteins to disrupt T-cell function could provide a selective advantage for the virus by reducing the ability of the host immune system to mount a response against the virus, a consequence being a reduction in the ability to mount other immune responses. The data presented here offer another possible mechanism by which SIV Nef contributes to immunodeficiency, and provide impetus for further analyses of the interactions of HIV-1 Nef proteins with the cell signalling machinery, interactions for which small molecule inhibitors could be developed.

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