Capacity of simian immunodeficiency virus strain mac Nef for high-affinity Src homology 3 (SH3) binding revealed by ligand-tailored SH3 domains

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The simian immunodeficiency virus (SIV) Nef protein contains a consensus Src-homology 3 (SH3) binding motif. However, no SH3-domain proteins showing strong binding to SIV Nef have yet been found, and its potential capacity for high-affinity SH3 binding has therefore remained unproven. Here we have used phage-display-assisted protein engineering to develop artificial SH3 domains that bind tightly to SIV strain mac (SIVmac) Nef. Substitution of six amino acids in the RT loop region of Hck-SH3 with the sequence E/DGWWG resulted in SH3 domains that bound in vitro to SIVmac Nef much better than the natural Hck- or Fyn-SH3 domains. These novel SH3 domains also efficiently associated with SIVmac Nef when co-expressed in 293T cells and displayed a strikingly differential specificity when compared with SH3 domains similarly targeted for binding to human immunodeficiency virus type 1 (HIV-1) Nef. Thus, SIVmac Nef is competent for high-affinity SH3 binding, but its natural SH3 protein partners are likely to be different from those of HIV-1 Nef.

Introduction

Nef is a 27–35 kDa accessory protein unique to the primate lentiviruses human immunodeficiency virus types 1 and 2 (HIV-1 and -2) and simian immunodeficiency virus (SIV). It is important for virus replication and the development of AIDS in HIV-1-infected humans as well as in SIV-infected macaques (Cullen, 1999; Figuet et al., 1999). Despite considerable sequence variation among different Nef alleles, amino acids that form a minimal consensus binding site (PxxP motif) for Src homology 3 (SH3) domains are highly conserved in lentiviral Nef sequences (Shugars et al., 1993; Los Alamos Database, http://hiv-web.lanl.gov). SH3 domains are small (typically 60 amino acids) modular protein domains that mediate protein interactions by binding to their PxxP motif-containing targets and are found in a large number (more than 200 in human) of proteins often involved in intracellular signalling and cytoskeletal organization (Dalgarno et al., 1997; Mayer, 2001).

The ability of Nef to modulate signal transduction pathways, as well as many other of its cellular functions, such as enhancement of HIV-1 replication and down-modulation of cell-surface expression of human leukocyte antigen class I (HLA-I), have been shown to depend on its SH3-binding (PxxP) motif (Figue et al., 1999; Renkema & Saksela, 2000; Geyer et al., 2001). Notably, AIDS-like pathology observed in CD4–HIV-1 Nef transgenic mice (Hanna et al., 1998) was found to be absent in mice similarly expressing a Nef transgene with a disrupted PxxP motif (Hanna et al., 2001). The relevant SH3 proteins involved in mediating these different functions of Nef remain incompletely characterized. SH3 domain-containing cellular binding partners of HIV-1 Nef reported to date include the Src family tyrosine kinases Hck, Lyn, Fyn and Lck and the adapter protein guanine exchange factor Vav (Renkema & Saksela, 2000). Breeding of CD4–HIV-1 Nef transgenic mice into an Hck–/– background has been reported and was found to delay, although not abolish, HIV-1 Nef-induced pathogenesis in this animal model (Hanna et al., 2001).

Binding of HIV-1 Nef to the Hck SH3 domain is relatively strong \((K_D \sim 2 \mu M)\), representing one of the tightest known naturally occurring SH3–ligand complexes (Lee et al., 1995). Detailed studies on this interaction have shown that most of the binding affinity in this case, and presumably in many other tight SH3–ligand complexes, is due to molecular contacts involving discontinuously positioned amino acid residues outside the primary SH3 domain docking site formed by the PxxP motif region (Lee et al., 1995; Manninen et al., 1998).
the SH3 surface, the matching contacts are provided by a structure known as the RT loop, a region that represents maximal sequence diversity among different SH3 domains (Lee et al., 1995, 1996). Despite the overall similarity of HIV-1 and SIV/HIV-2 Nef proteins, the role of SH3 binding in mediating the cellular functions of SIV/HIV-2 Nef remains unclear. Although SIV Nef and HIV-1 Nef share the ability to interact with Src family tyrosine kinases, in the case of SIV Nef these interactions appear to be predominantly SH3-independent (Greenway et al., 1999). Moreover, while both HIV-1 and SIV Nef can down-regulate HLA class I cell-surface expression, only HIV-1 Nef is dependent on its PxxP motif in this function (Swigut et al., 2000). Experimental infection of macaques with isogenic SIV strains carrying wild-type or PxxP-mutated viruses has yielded conflicting data. P. A. Luciw and colleagues concluded that this motif is critical for pathogenesis of simian AIDS (Khan et al., 1998). However, studies by F. Kirchhoff and co-workers have indicated that although mutations introduced to disrupt the SIV Nef PxxP motif are under pressure to revert, such reversions typically occur late during disease progression (Lang et al., 1997; Carl et al., 2000), suggesting that SH3 binding may play only a limited role in the in vivo functions of SIV Nef.

While many SH3 domains, such Hck and Fyn, show some affinity for SIV Nef (Greenway et al., 1999; Collette et al., 2000), so far no SH3 domain has been identified that will bind SIV Nef with high affinity. This is unlikely to be caused by a failure to test binding of SIV Nef with the relevant simian orthologues of human SH3-binding proteins that interact with HIV-1 Nef. For example, Picard et al. (2002) have recently cloned simian Hck, which was found to be 97.8% identical overall and 100% identical in its SH3 region with human Hck; nevertheless, it was not any better than its human counterpart in binding to SIV Nef. Moreover, protein engineering studies by Collette et al. (2000) have shown that if the SH3 RT loop-accommodating amino acid residues from HIV-1 Nef are introduced into the corresponding positions in SIV Nef, binding to Src family SH3 domains can be significantly improved. Thus, the observed low SH3-binding capacity of SIV Nef might be due to poorly matching RT loop regions in the Src family and other SH3 domains tested so far. Nevertheless, in light of the studies introduced above, the question arises as to whether SIV Nef is at all competent for high-affinity SH3 binding.

In this study we have addressed this problem by taking advantage of a strategy that we have previously developed to generate artificial SH3 domains showing strong binding to HIV-1 Nef (Hiipakka et al., 1999) for use as intracellular inhibitors of HIV-1 Nef (Hiipakka et al., 2001). This strategy is based on phage-display libraries presenting Hck-derived modified SH3 domains that carry random sequences in place of six amino acid residues forming the non-conserved region of the RT loop of Hck-SH3. Here we report successful development of novel SH3 domains that bind tightly to SIVmac Nef. In addition to providing further support for the applicability of our strategy for generating SH3 domains with tailored binding properties, these results clearly show that SIVmac Nef has a capacity for high-affinity SH3 binding.

Methods

Phagemids and plasmids. The multivalent phagemid vector pG8H6 (Jacobsson & Frykberg, 1996) was a gift from Lars Frykberg (Uppsala, Sweden). The monovalent phagemid pCANTAB-5E vector was purchased from Amersham Biosciences. Construction of the pGEX vectors for bacterial expression of GST fusion proteins for the SH3 domains has been described previously (Hiipakka et al., 1999; Saksela et al., 1995). To express the SIVmac Nef and HIV-1 Nef NL4-3 RT1 (Saksela et al., 1995) in mammalian cells, the corresponding cDNAs were cloned into the pEBB expression vector (Mizushima & Nagata, 1990) provided with a Myc epitope tag. The SH3 domains E and D were inserted as GST fusions into the pEBB-myr-HA vector, as previously described for the HIV-1 Nef-specific clones A1 and B6 (Hiipakka et al., 2001).

Phage library construction and affinity selection. The multivalent library of artificial Hck-derived SH3 domains (RTT-SH3s) was created in pG8H6 using a degenerate PCR primer, as previously described for pCANTAB-5E (Hiipakka et al., 1999). In addition, a TAG stop codon was inserted in front of the RTT-SH3 sequence to reduce expression of the fusion protein using a supE host (TG1) in which amber termination is incompletely suppressed. A total of 4.2×10⁸ individual recombinant colonies were obtained by electroperation into TG1 cells and used to produce recombinant phage preparations via infection with the M13KO7 helper phage (Hiipakka et al., 1999). Sublibraries of approximately 500,000 recombinant clones consisting of PCR-amplified RTT-SH3 inserts initially selected using the multivalent system were inserted into pCANTAB-SEP, as previously described (Hiipakka et al., 1999). Affinity selection of clones from the multi- and monovalent libraries was also carried out as described previously (Hiipakka et al., 1999).

Cell culture, transfections and reporter gene assays. 293T and Jurkat (JE-6) cells were obtained from ATCC and cultured using standard procedures. 293T cells were transfected using the Lipofectamine reagent (Invitrogen) and the JE-6 cells using DMRIE-C transfection reagent (Invitrogen), as previously described (Manninen et al., 2000). The reagents and protocols used for the luciferase assays have also been described previously (Hiipakka et al., 2001).

In vitro binding experiments. Expression and purification of bacterial GST–SH3 fusion proteins was carried out as previously described (Hiipakka et al., 1999). SIVmac and HIV-1 Nef protein-containing lysates were produced by transient transfection of 293T cells. Approximately 2×10⁷ transfected cells were collected 48 h after transfection and lysed in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.5 mM sodium deoxycholate, 50 mM NaF, 1 mM PMSF and 10 μg/mL aprotinin). The lysates were serially diluted (fourfold dilutions) into a similarly prepared lysate of untransfected 293T cells. After removal of small aliquots to monitor their Nef content, 200 μl of these dilutions were incubated for 2 h at 4 °C with glutathione-Sepharose 4B beads (Amersham Biosciences) coated with 8 μg plain GST or GST–SH3 proteins. After the incubation, the beads were washed three times with PBS and boiled in SDS–PAGE sample buffer and analysed by SDS–PAGE to examine the amount of GST–SH3 and associated Nef proteins bound to the beads following immunoblotting with anti-HA and anti-Nef antibodies.
anti-Myc antibodies, respectively, and subsequent detection by the enhanced chemiluminescence (ECL) system, as suggested after the manufacturer (Amer sham Biosciences).

**In vivo binding experiments.** Forty-eight hours after transfection with expression vectors for the GST–SH3 and Nef proteins, 293T cells were harvested, washed with PBS and lysed into 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl₂, 10 mM NaF, 1 mM Na-orthovanadate, 1 mM PMSF and 10 μg/ml aprotinin. Lysates (1 mg total cellular protein) were incubated with glutathione–Sepharose 4B beads for 4 h at 4 °C. After three washes with PBS, the proteins associated with the beads were examined by SDS–PAGE followed by immunoblotting with anti-Myc and anti-HA antibodies and detection by ECL. Thirty μg from each lysate was removed before addition of the beads to verify uniform expression of the transfected GST–SH3 and Nef proteins.

**Results and Discussion**

To examine whether SH3 domains capable of high-affinity binding to SIVmac Nef could be generated by modifying the RT loop region of Hck-SH3, a multivalent phage library expressing RRT-SH3 domains was subjected to affinity selection using recombinant SIVmac Nef protein. After five (experiment 1) or seven (experiment 2) rounds of selection and helper virus-assisted amplification, 12 individual RRT-SH3 clones were isolated and sequenced. In addition, a pool of inserts of recombinant multivalent phages from the second and third round of selection were cloned into a monovalent phagemid vector in order to carry out further selection rounds under these more stringent conditions where typically only one RRT-SH3 molecule is displayed per phage particle. After four additional rounds of selection in the monovalent system, 10 individual RRT-SH3 clones were sequenced. However, both selection strategies resulted in the exclusive enrichment of two related sequences, EGWWG and DGWWG, which in the following are referred to as E and D. Neither of the SIVmac Nef-selected clones contained any of the sequence motifs previously found in the HIV-1 Nef-selected RRT-SH3 domains (Hiipakka et al., 1999), nor did they resemble RT loop regions of natural SH3 domains in the GenBank database.

Selection of the sequences EGWWG and DGWWG from the library of Hck-derived SH3 domains designed to carry six randomized RT loop residues suggested a strong selective advantage for a shorter RT loop in binding to SIVmac Nef. Possibly the original library contained a sufficiently large collection of clones with five-residue substitutions from which these sequences were selected, or, alternatively, they may have evolved via a deletion during the phage selection/re-amplification process. In either case, this appears to be specific for SIVmac Nef, since the length of six modified RT loop residues has been strictly conserved among the large number of RRT-SH3 domains binding with high affinity to HIV-1 Nef that we have selected previously (Hiipakka et al., 1999). It should be noted that the RT loop region in various natural SH3 domains is not fixed to six residues but, in addition to sequence diversity, also shows considerable variation in length.

To characterize further the ability of the phage display-selected SH3 clones E and D to bind to SIVmac Nef, they were expressed as GST fusion proteins in *E. coli* and used to precipitate lysates of human 293T cells transfected with an SIVmac Nef expression vector. Native Hck-SH3 as well as Fyn-SH3, which has been implicated as the natural SH3 domain with most affinity for SIV Nef (Collette et al., 2000), were similarly expressed as GST fusion proteins and tested in parallel with E and D. For comparison, HIV-1 Nef-transfected 293T cell lysates were also used in order to include the HIV-1 Nef/Hck-SH3 interaction with the known affinity of K_D 250 nM (Lee et al., 1995) as an internal control.

Equal amounts of the GST–SH3 fusion proteins of Hck, Fyn, D and E were coated on glutathione–Sepharose beads (Fig. 1a) and incubated with Nef-expressing lysates serially diluted with lysates of untransfected 293T cells (Fig. 1b). The amount of Nef proteins bound to the different GST–SH3 proteins after washing of the beads is shown in Fig. 1(c). The SIVmac Nef-selected clones E and D precipitated readily detectable amounts of SIVmac Nef, even from the 64-fold diluted lysate (Fig. 1c). In contrast, the affinity of Hck-SH3 and Fyn-SH3 was too weak to bring down enough SIVmac Nef to be visible, even in very long exposures of the immunoblots (Fig. 1c and data not shown). Thus, we concluded that the EAIHHE to E/DGWWG modification in the Hck-SH3 RT loop was associated with a very significant, apparently at least two orders of magnitude, improvement in binding to SIVmac Nef. Unlike SIVmac Nef, HIV-1 Nef could be precipitated under the same experimental conditions by the natural Hck-SH3, as expected based on the known affinity of this interaction (K_D 250 nM). Although the assay was not strictly quantitative, the ability of E and D to precipitate from a 64-fold diluted lysate an amount of SIVmac Nef comparable with HIV-1 Nef precipitated by Hck-SH3 from an undiluted lysate suggested that the affinity of E and D binding to SIVmac Nef was probably in the low nanomolar range.

To confirm that the SH3 domains E and D could also associate with SIVmac Nef in living cells, they were subcloned into a mammalian expression vector, in which they were expressed as N-terminally myristoylated GST fusion proteins containing a C-terminal HA-epitope tag. The same strategy has previously been used to engineer HIV-1 Nef-specific RRT-SH3 domains for intracellular capture and inhibition of function (Hiipakka et al., 2001). Two such HIV-1 Nef proteins (clones A1 and B6; Hiipakka et al., 2001) were also included in the present experiment for comparison. 293T cells expressing either SIVmac or HIV-1 Nef together with one of these RRT-SH3 vectors or a control vector expressing similarly modified plain GST were lysed and the GST–SH3 fusion proteins (or GST alone) together with the Nef proteins complexed with them were bound to glutathione–Sepharose beads. As shown in Fig. 2(a), equal levels of different GST–SH3 proteins as well as HIV-1 and SIVmac Nef proteins were present in the unselected lysates. Accordingly, equal amounts of the GST–
Fig. 1. RRT-SH3 proteins associate with SIVmac Nef in vitro. (a) Glutathione–Sepharose beads coated with recombinant GST–SH3 proteins used for precipitation of Nef from lysates of SIVmac or HIV-1 Nef-transfected 293T cells in (c) were examined to confirm the integrity and even loading of the GST–SH3 proteins by SDS–PAGE and Coomassie blue staining. (b) The amount of SIVmac and HIV-1 Nef proteins in the lysates used for the pull-down assay in (c) was examined by Western blotting using an antibody against the Myc epitope present in the C terminus of the transfected Nef proteins. The lysates were prepared by four-fold dilution (up to 1:64) of the SIVmac or HIV-1 Nef-expressing lysates into a corresponding lysate of untransfected cells. A volume of each lysate dilution corresponding to one-tenth of that incubated with the different SH3-containing beads was loaded onto the gel. (c) The amount of Nef captured by the indicated GST–SH3 proteins from the serially diluted Nef-transfected lysates was analysed after several washes by immunoblotting as in (b). The combination of the lysate and the SH3 domain used for pull-down is indicated to the right of each of the blots, which were processed in parallel and under identical conditions.

Fig. 2. RRT-SH3 proteins associate with SIVmac Nef in vivo. Lysates of 293T cells transfected with vectors expressing either SIVmac or HIV-1 Nef (both Myc-tagged) together with expression vectors for different HA-tagged GST–SH3 fusion proteins (or GST alone) were examined by immunoblotting using anti-Myc and anti-HA antibodies either directly from total lysates (a) or after capture of the GST–SH3 and associated Nef proteins with glutathione–Sepharose beads from precipitates (b). SH3 proteins were also captured from these lysates by glutathione–Sepharose beads (Fig. 2b, upper panel). In contrast, the amount of co-precipitating Nef varied significantly. SIVmac

Fig. 3. Co-expression of specific RRT-SH3 domains can inhibit Nef-induced NFAT activation. Jurkat cells were transfected with an NFAT-responsive reporter plasmid and different SH3 expression vectors, as indicated, together with SIVmac Nef (black bars) or HIV-1 Nef (white bars). After 20 h the transfected cells were stimulated for 4 h with PMA followed by luciferase activity measurement. In both cases, Nef-induced NFAT transcriptional activation was set to 100% and the luciferase activity in SH3-transfected cells is shown relative to this. Mean luciferase activities from three independent transfections are shown. Standard error bars indicate inter-assay variation.
Nef was readily precipitated by D and E, but not at all by A1 and B6, which thus appeared specific for HIV-1 Nef (Fig. 2b, lower panel). On the other hand, in addition to their engineered ability to bind efficiently to SIVmac Nef, D (Fig. 2b, lower panel) and to a lesser extent E (evident only in longer exposures of the blot; data not shown) showed some association with HIV-1 Nef.

To examine the effect of binding of E and D on SIVmac Nef function and to exclude the theoretical possibility that the complexes shown in Fig. 2 might have been formed only after the transfected cells were lysed, we also studied the ability of E/D co-transfection to inhibit SIVmac Nef-mediated activation of the transcription factor NFAT in Jurkat T cells. In agreement with our previous findings (Manninen et al., 2000, 2001), when HIV-1 Nef was expressed in the absence of an inhibitory SH3 domain (a control GST vector was used instead), both HIV-1 and SIVmac Nef induced NFAT activity, as judged by an increase in the expression of an NFAT-responsive reporter gene in phorbol ester-stimulated Jurkat cells. Luciferase expression in HIV-1 Nef expressing cells was 20-fold higher and in SIVmac Nef-expressing cells sevenfold higher than in cells not transfected with Nef (data not shown). These values were adjusted to 100% for both in Fig. 3 to facilitate comparison of the effects of co-transfected SH3 expression constructs. As shown in Fig. 3, D and E were indeed able to inhibit SIVmac Nef-induced NFAT activation efficiently, and, in agreement with the co-precipitation data (Fig. 2b and data not shown), also showed some inhibition of HIV-1 Nef-activated NFAT-dependent gene expression. In contrast, despite being strong inhibitors of HIV-1 Nef, A1 (RT loop sequence VSWSPD instead of D/EGWGG) did not interfere with SIVmac Nef function and B6 (RT loop YSPFSW) only marginally inhibited SIV Nef in this assay. This result is also in good agreement with the interaction data and provides a striking demonstration of the degree of differential binding specificity that can be built into a single SH3 domain by manipulating the RT loop.

Taken together, the data reported here show that SH3 domains capable of high-affinity binding to SIV Nef can be developed. Therefore, the lack of success experienced in attempting to identify cellular SH3 proteins that would bind strongly to SIV Nef does not reflect a failure of SIV Nef to properly accommodate an SH3 domain, for example due to poor presentation of its PxxP motif. Although the available data suggest that the role of SH3 binding in mediating the intracellular functions of SIV Nef may be less critical than in the case of HIV-1 Nef (Swigut et al., 2000; Lang et al., 1997; Carl et al., 2000), the conservation of the PxxP motif in Nef proteins encoded by many divergent strains of SIV suggests that an as yet unidentified cellular SH3 protein(s) capable of interacting with SIV Nef probably exists. The biological role or relevance of such Nef/SH3 complexes in the virus–host interactions operative in SIV infections is not clear, particularly when considering the asymptomatic nature of these infections in their natural hosts. Nevertheless, based on the distinct preferences in SH3 RT loop selection shown in this study, it can be predicted that the putative SIV Nef-associated SH3 proteins are likely to be structurally and functionally different from those utilized by HIV-1 Nef.

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