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In vitro binding and phosphorylation of human immunodeficiency virus type 1 Nef protein by serine/threonine protein kinase

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Although the human immunodeficiency virus type 1 (HIV-1) *nef* gene still has no precisely defined function, *in vivo* studies have demonstrated that Nef is an important pathogenic determinant of HIV. In order to identify cellular proteins capable of binding to Nef, the HIV-1_{LAI} *nef* gene product was expressed in the bacterial vector pGEX-2T as a glutathione *S*-transferase (GST)–Nef fusion protein. Deletion mutants corresponding to 86 and 35 N-terminal residues of the Nef protein were prepared. The GST–Nef constructs were used to identify cellular kinases capable of interacting with Nef. After incubation with a Jurkat cell lysate, the GST–Nef constructs immobilized on glutathione–agarose beads bound to cellular kinase(s) and were phosphorylated at three sites *in vitro*: one on threonine at position 15, one on serine between residues 1 and 35, and one on threonine between residues 36 and 86. The Nef-phosphorylating activity was inhibited by protein kinase C (PKC)-selective inhibitors. Cell fractionation showed that this Nef-binding kinase was mainly in the membrane-associated fraction. These results suggest that kinase(s) of the PKC family are specifically bound to and phosphorylate Nef *in vitro*. The interaction of Nef with cellular kinases and its phosphorylation may be important in mediating the effects of Nef in HIV-1 pathogenesis.

Introduction

Nef is encoded by an open reading frame located at the 3′ end of the human immunodeficiency virus type 1 (HIV-1) genome. Nef is one of the most controversial HIV proteins. It was initially reported to mediate the down-regulation of HIV replication in cultured cells (Luciw *et al.*, 1987; Terwilliger *et al.*, 1986); these findings were later not confirmed or contradicted (Hammes *et al.*, 1989; Kim *et al.*, 1989; Bachelerie *et al.*, 1990; de Ronde *et al.*, 1992; Chowers *et al.*, 1994). Numerous results suggest that the *nef* gene is not necessary for viral replication *in vitro*. In contrast, *in vivo* experiments indicate that *nef* plays an essential role in natural infection (Kestler *et al.*, 1991; Jamieson *et al.*, 1994). Several cellular targets, including CD4 (Guy *et al.*, 1987; Garcia & Miller, 1991), interleukin-2 (Luria *et al.*, 1991) and the transcription factors NF-κB and AP-1 (Bandres & Ratner, 1994; Niederman *et al.*, 1992, 1993) all appear to be down-regulated by Nef. Recently Nef has also been associated with the reduced cell surface expression of the processed HIV-1 glycoprotein (Schwartz *et al.*, 1993). Autophosphorylation of Nef in the presence of GTP and/or ATP has been described (Guy *et al.*, 1987, 1990; Nebrada *et al.*, 1991). However, there have been conflicting reports about Nef autokinase activity and about its putative nucleotide-binding or GTPase activities (Kaminchik *et al.*, 1990; Backer *et al.*, 1991; Matsuura *et al.*, 1991; Harris *et al.*, 1992). Nef has also been found to be phosphorylated by protein kinase C (PKC) on the threonine at position 15 (Guy *et al.*, 1987). This phosphorylation has been detected *in vitro* by a kinase assay and in cells stably transfected with Nef expression vectors, but has not yet been detected in HIV-1-infected cells. The significance of this phosphorylation is not clear and a significant rate of mutation (mostly Thr to Ala) at this site has been described in viruses isolated from cultured cells (Laurent *et al.*, 1990) and from HIV-1-infected patients (Delassus *et al.*, 1991). Recent studies suggest that Nef may be associated with a kinase activity (Poulin & Levy, 1992; Sawai *et al.*, 1994) and the phosphorylation of Nef by PKC and/or other cellular kinases could be functionally important *in vivo* as it might regulate Nef activity (Bandres *et al.*, 1994).

This report describes the *in vitro* binding of cellular protein kinase(s) to HIV-1_{LAI} Nef protein, expressed as a

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glutathione *S*-transferase (GST) fusion, and its subsequent phosphorylation. By using Nef deletion mutants we detected three sites of phosphorylation *in vitro*: one on Thr-15, one on serine residue(s) in the N-terminal part of the protein between amino acid (aa) 1 and aa 35, and one on threonine residue(s) between aa 36 and aa 86. The results suggest that the kinase responsible is a member of the PKC family which specifically binds to and phosphorylates Nef. An unidentified 64 kDa protein that is phosphorylated by a PKC-independent mechanism was also found to be specifically bound to Nef.

Methods

Generation of recombinant pGEX-2T-nef plasmids. The *nef*-coding sequence (621 bp) was recovered using PCR on the whole genome of the HIV-1_{LAI} strain (Peden *et al.*, 1991). The oligonucleotide primers used for amplification of the *nef* gene were designed as follows (upper case letters indicate HIV-1 sequences, lower case letters indicate the restriction enzyme sites): 5′ primer, (*Bam*HI) cgggatccATGGGTGGCAAGTGGTCAAAAAG (HIV-1_{LAI} positions 8390–8412); 3′ primer, (*Eco*RI) ccggaattcTCAGCAGTTCTTGAAGTACTCCG (HIV-1_{LAI} 8988–9010). The PCR products were purified by gel electrophoresis, digested with *Bam*HI and *Eco*RI, and cloned into the *Bam*HI–*Eco*RI sites of the bacterial expression vector pGEX-2T (Pharmacia), in frame with the GST gene. Double-stranded DNA sequencing was carried out using a Sequenase version 2.0 kit (United States Biochemical) according to the protocol provided by the manufacturer, to check the insertion and to insure that PCR did not introduce any errors into the sequence. To generate *nef* deletion mutants (GST-*nef* Δ mutants) the recombinant pGEX-2T-*nef* was digested with *Xho*I and *Eco*RI for pGEX-2T-*nef* X/E (deletion 36–206), or with *Bgl*II and *Eco*RI for pGEX-2T-*nef* B/E (deletion 87–206)

Expression, immobilization and purification of the GST-Nef fusion proteins. The GST, GST–Nef and GST–Nef Δ mutants were expressed, immobilized and purified essentially as previously described (Bougeret *et al.*, 1993; Jullien *et al.*, 1994). *Escherichia coli* (DH5α; Gibco BRL) transformed either with pGEX-2T, pGEX-2T-*nef* or pGEX-2T-*nef* Δ mutants was grown overnight at 37 °C in Luria-Bertani medium (LB) containing 50 μg/ml ampicillin (Sigma), diluted 1:10 in LB, and incubated at 37 °C for 1 h. Protein expression was induced by adding 0·1 mM-IPTG (Clontech Laboratories) and growth was continued at 37 °C for 5 h. Bacteria were harvested and resuspended in TENGN buffer (50 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 100 mM-NaCl, 0·1% NP40 and 10% glycerol) containing protease inhibitors (1 mM-PMSF, 2% aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin) and 1 mM-DTT. The bacteria were lysed on ice by adding 0·5 mg/ml lysozyme (Boehringer Mannheim) for 30 min followed by sonication and centrifugation at 10000 *g* for 15 min at 4 °C. The supernatant (containing soluble proteins) was divided into aliquots and stored at −80 °C. Soluble proteins were incubated with glutathione (GSH)-agarose beads for 30 min at room temperature; the beads were then washed three times with 1 M-NaCl and twice with PBS. The amount of fusion protein immobilized on the beads was estimated after SDS–PAGE and Coomassie blue staining by comparing the intensities of bands with those of proteins standard of known concentration.

Preparation of a total Jurkat cell extract. Jurkat human T cells (clone J77–3.8) were suspended (2×10^7 cells/ml) in TEN buffer (10 mM-Tris–HCl pH 7·2, 5 mM-EDTA, 150 mM-NaCl) supplemented with 0·1 mM-sodium orthovanadate, 1% NP40, 1% aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM-PMSF (TEN^+ buffer). The cells were lysed on ice for 30 min and then centrifuged for 10 min at 10000 *g*. The supernatant (Jurkat cell extract) was divided into aliquots and stored at −80 °C.

Preparation of cytosolic and membrane fractions of Jurkat cell extract. Jurkat cells were washed in cold PBS and suspended in TEN^+ buffer without NP40 (6×10^7 cells/ml). The cells were then cavitated in a nitrogen bomb at 250 p.s.i. at 4 °C and the homogenate was centrifuged to remove debris, intact cells and nuclei. The supernatant was ultracentrifuged at 200000 *g* for 1 h at 4 °C (Beckman TL100). The supernatant (cytosolic fraction) was divided into aliquots and stored at −80 °C. The pellet was suspended in 1 ml TEN^+ buffer, incubated on ice for 30 min and clarified by centrifugation for 5 min at 15000 *g*. The supernatant (membrane fraction) was divided into aliquots and stored at −80 °C. The protein content of cytosolic and membrane fractions was estimated by SDS–PAGE after Coomassie blue staining.

In vitro binding of GST, GST-Nef and GST-Nef Δ mutants to Jurkat cell extract proteins. A Jurkat cell extract (40 μl; equivalent to 8×10^5 cells) was incubated for 2 h at 4 °C with approximately 5 μg of each recombinant protein coupled to GSH–agarose beads. The beads were then washed at 4 °C in 10 mM-Tris–HCl pH 7·2, 5 mM-EDTA, 1% Triton X-100, 1% sodium deoxycholate, 10 mM-sodium orthovanadate and three concentrations of NaCl, 300 mM (for three washings), 150 mM (for two washings) and 10 mM (one washing).

The binding of cellular kinases from cytosolic or membrane fractions to GST–Nef or GST–Nef Δ mutants was assayed by testing 40 μl of each fraction (equivalent to $2{\cdot}5 \times 10^6$ cells) as previously described for the Jurkat cell extract.

In vitro phosphorylation of GST, GST-Nef and GST-Nef Δ mutants bound to proteins from a Jurkat cell extract. The fusion proteins immobilized on the beads that had been incubated with Jurkat cell extract (as described above) were washed in phosphorylation buffer [50 mM-PIPES pH 7, 10 mM-$MnCl_2$, 10 mM-$MgCl_2$, 0·1 mM-sodium orthovanadate (as phosphatase inhibitor), 1 mM-PMSF, 2% aprotinin]. Phosphorylation buffer (20 μl) containing 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol; Du Pont-NEN) was added to the immobilized proteins and incubation was carried out for 10 min at 30 °C. The reaction was terminated by adding SDS-sample buffer. The proteins were resolved by SDS–PAGE and the phosphorylated proteins were detected by autoradiography at −80 °C on X-ray film (Amersham).

Phosphorylation experiments were also performed as described above, but in the presence of the PKC inhibitors GF 109203X (0·01 or 0·02 mM; Glaxo; Toullec *et al.*, 1991) and isoquinoline H7 (0·05 mM; Hidaka *et al.*, 1984).

In experiments where Nef was used as an exogenous substrate, the reactions were performed as described above except that 3 μg Nef was added to the kinase reaction mix. Purified Nef was obtained after cleavage of GST–Nef with thrombin (Bougeret *et al.*, 1993). Absence of residual thrombin activity was verified by incubating phosphorylated GST–Nef with Nef.

Phosphoamino acid analysis. Phosphoamino acids were analysed by the method of Kamps & Sefton (1989). Five μg of the fusion proteins (GST–Nef, GST–Nef X/E and GST–Nef B/E) labelled *in vitro* with [γ-^{32}P]ATP, as described above, were resolved by SDS–PAGE and transferred to Immobilon PVDF membrane (Millipore). The bands containing the fusion proteins were cut out and incubated with 100 μl of constantly boiling 6 M-HCl (Sigma) for 1 h at 110 °C. Phosphoamino acids were analysed on thin-layer cellulose plates (Schleicher & Schuell) by two-dimensional electrophoresis at pH 1·9 in the first dimension and at pH 3·5 in the second dimension (Hunter & Sefton, 1980) using a HTLE-7000 electrophoresis unit (C. S. B. Scientific). Standard phosphoamino acids were identified by staining with 0·25% ninhydrin in acetone. Autoradiography was then performed to detect γ-^{32}P-labelled phosphoamino acids.

Results

Expression, immobilization and purification of GST, GST–Nef and GST–Nef Δ mutants

The prokaryotic GST expression system was used to produce recombinant Nef or Nef Δ mutants as GST fusion proteins (Smith & Johnson, 1988). The constructs are shown in Fig. 1; pGEX-2T-*nef* was obtained by inserting the whole *nef* coding sequence amplified by PCR. Deletion mutants were obtained by cleaving pGEX-2T-*nef* at different restriction sites to give pGEX-2T-*nef* B/E (Δ aa 87–206), corresponding to the 86 N-terminal aa, and pGEX-2T-*nef* X/E (Δ aa 36–206), corresponding to the 35 N-terminal aa. The recombinant proteins were purified in a one-step procedure on GSH–agarose beads. The GST-coupled proteins were analysed by SDS–PAGE (Fig. 2*a*). Recombinant fusion proteins with the expected molecular masses were obtained: GST–Nef (50 kDa), GST–Nef B/E (35 kDa) and GST–Nef X/E (30 kDa) (Fig. 2*a*, lanes 1–3, respectively). GST–Nef was immobilized as essentially pure, full-length fusion protein. But the truncated forms GST–Nef B/E and GST–Nef X/E, although mainly consisting of the full-length proteins, also included degradation products of lower molecular masses, including GST. As a control, GST was produced and immobilized on GSH–agarose beads and purified in parallel with the Nef fusion proteins (Fig. 2*a*, lane 4). Western blot analysis, using either a rat anti-Nef monoclonal antibody (BF7) or a mouse anti-GST monoclonal antibody (104-1), was used to confirm the expression of the GST–Nef protein (data not shown).

Cellular protein kinase(s) bind to and phosphorylate Nef in vitro

We first demonstrated that there was no autophosphorylation of GST–Nef or Nef (after cleavage of GST–Nef with thrombin) or of GST (data not shown).

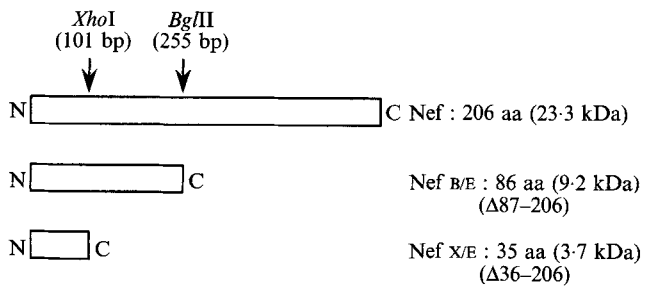

Fig. 1. Constructs for expressing intact and deleted Nef in bacteria. Full-length or deleted *nef* DNA fragments were inserted into the bacterial pGEX-2T expression plasmid to generate Nef recombinants with various C-terminal deletions. The positions of aa, deleted fragments (Δ) and the molecular masses of the expected proteins are indicated.

Then, 5 μg of each of the fusion proteins immobilized on GSH–agarose beads were incubated with Jurkat cell extract to determine whether GST–Nef and GST–Nef Δ mutants were bound to and phosphorylated by cellular protein kinases. The beads were washed extensively under stringent conditions and an *in vitro* phosphorylation assay was performed with [γ-^{32}P]ATP, assuming that cellular protein kinases were retained by the immobilized GST–Nef constructs. GST–Nef, GST–Nef B/E and GST–Nef X/E, but not GST, were phosphorylated under these conditions (Fig. 2*b*, lanes 1–4, respectively). The full-length protein of the truncated form of Nef, GST–Nef X/E (Fig. 2*b*), and a lower molecular mass degradation product were phosphorylated, while the smaller degradation product (probably GST) was not phosphorylated. The mutants GST–Nef X/E and GST–Nef B/E were phosphorylated much more efficiently than full-length GST–Nef. Conformational changes in Nef Δ mutants could facilitate binding of the kinase and/or lead to better accessibility of the phosphorylation sites occluded in the full-length molecule. Moreover, in the absence of any phosphopeptide mapping data it is not possible to conclude that the same sites are being phosphorylated in all three proteins. When the phosphorylated GST–Nef was treated with thrombin (Fig. 2*c*), only Nef appeared to be phosphorylated, confirming that the substrate of the kinase was indeed the Nef portion of the fusion protein rather that GST.

We checked that this kinase activity was specifically retained on the Nef portion of the fusion protein (and not on GST or on the beads) by running kinase assays as described above, except that 3 μg recombinant Nef was added to the reaction mixture as exogenous substrate. GST did not bind the kinase, since the exogenous Nef was not phosphorylated (Fig. 2*d*, lane 1). But exogenous Nef was strongly phosphorylated when GST–Nef was used (Fig. 2*d*, lane 2).

A band of slightly lower mobility (between 31 kDa and 45 kDa) was observed during GST–Nef phosphorylation (Fig. 2*b*, *c*, *d*). This band, weakly visible by Coomassie blue staining (Fig. 2*a*), was probably a GST–Nef degradation product since it was recognized in Western blotting by both anti-Nef (BF7) and anti-GST (104-1) monoclonal antibodies (data not shown).

Phosphorylated proteins bound to the immobilized fusion proteins were also detected. Most of them were also visible in the control GST autoradiograph after a longer exposure. However, a 64 kDa phosphorylated protein (p64) was specifically bound to the GST–Nef constructs and not to the control GST (Fig. 2*b*, lane 4). Similar to the observation that the Nef Δ mutants were more phosphorylated than intact Nef, the p64 Nef-associated protein was found to bind strongly to GST–Nef B/E and GST–Nef X/E (Fig. 2*b*, lanes 2 and 3,

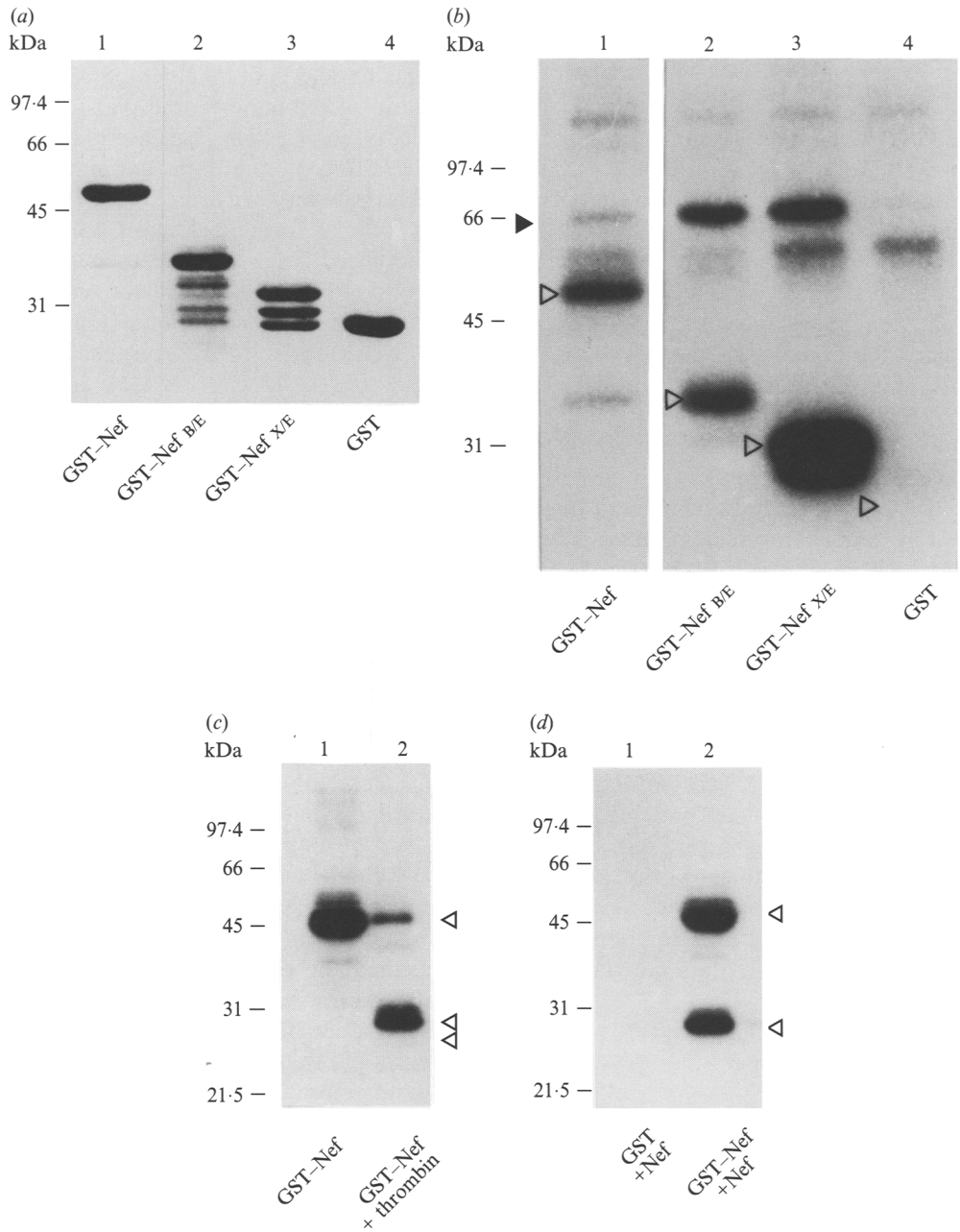

Fig. 2. Expression and *in vitro* phosphorylation of GST, GST–Nef and GST–Nef Δ mutants.(*a*) The expressed recombinant proteins were purified in a one-step procedure and immobilized on GSH–agarose beads. Washed beads (5 μl of a 50% v/v suspension in PBS) were analysed by 12% SDS–PAGE followed by Coomassie blue staining. (*b*) *In vitro* phosphorylation of GST, GST–Nef and GST–Nef Δ mutants. GSH–agarose-immobilized protein (5 μg) was incubated with Jurkat cell lysate, washed extensively and the immobilized proteins were phosphorylated *in vitro* by incubation for 10 min at 30 °C with [γ-^{32}P]ATP. The phosphorylated proteins were resolved by 12% SDS–PAGE and detected by autoradiography. Phosphorylated GST–Nef proteins (open arrows) and the p64 phosphorylated protein that interacted with all GST–Nef constructs (closed arrow) are indicated. (*c*) The binding and kinase reactions were performed with GST–Nef (lane 1) as described. The sample in lane 2 was subsequently treated with 1 unit of thrombin for 45 min at room temperature. The arrowheads mark the position of GST and Nef following cleavage with thrombin. (*d*) GST and GST–Nef were incubated with Jurkat cell lysate as described above. Kinase reactions were performed with 3 μg Nef as an exogenous substrate. The arrowheads mark the position of GST–Nef and Nef.

respectively). The p64 was less firmly bound to GST–Nef (Fig. 2*b*, lane 1), even when equimolar amounts of each fusion protein were used. Conformational changes of the deletion mutants leading to better accessibility of p64-binding sites could explain such differences.

The alkaline lability of both phosphorylated GST–Nef

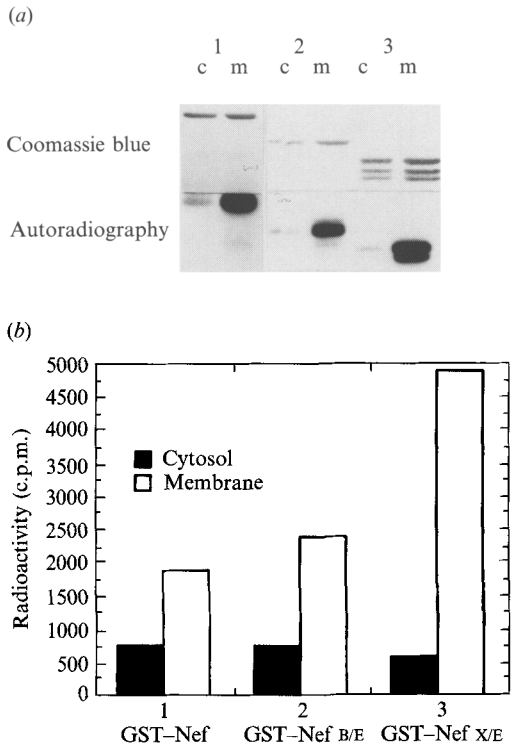

Fig. 3. *In vitro* phosphorylation of GST–Nef and GST–Nef Δ mutants by membrane and cytosolic fractions from Jurkat cells. (*a*) The GST–Nef proteins (lane 1, GST–Nef; lane 2, GST–Nef B/E; lane 3, GST–Nef X/E) were incubated with cytosol (c) or membrane (m) fractions of Jurkat cell extract, washed thoroughly and the immobilized proteins were phosphorylated *in vitro* in the presence of [γ-^{32}P]ATP. The proteins were then separated by 12% SDS–PAGE, stained with Coomassie blue and autoradiographed. (*b*) The labelled fusion proteins were excised from the gel and the incorporated radioactivity was counted.

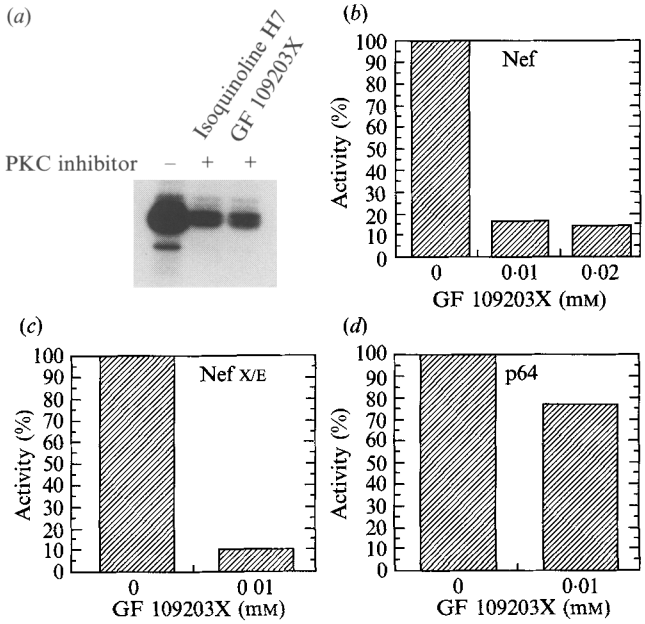

Fig. 4. Inhibition of Nef phosphorylation by selective PKC inhibitors. (*a*) GST–Nef was incubated with Jurkat cell extract as described in Methods and the kinase activity of the bead complexes was assayed without (–) and with (+) two PKC inhibitors (0·02 mM GF 109203X and 0·5 mM H7). The proteins were separated by 12% SDS–PAGE and the dried gel was autoradiographed. (*b*) GST–Nef and GST–Nef X/E were incubated with Jurkat cell extract and kinase activity was assayed without and with the selective PKC inhibitor GF 109203X (0·01 mM or 0·02 mM). The labelled proteins (GST–Nef, GST–Nef X/E and p64) were excised from the gel and the incorporated radioactivity was counted.

and p64 proteins suggested that these phosphorylation reactions occurred at serine and/or threonine residues (data not shown).

Characterization of the protein kinases associated with Nef

The subcellular location of the kinase(s) responsible for phosphorylating Nef was determined using cytosolic and membrane fractions from Jurkat cells. Immobilized GST–Nef and GST–Nef Δ mutants were incubated with these fractions, washed extensively and the fusion proteins phosphorylated with [γ-^{32}P]ATP *in vitro*. The proteins retained on the beads were separated by SDS–PAGE and stained with Coomassie blue (Fig. 3*a*). Phosphorylated proteins were detected by autoradiography (Fig. 3*a*). Finally, the bands corresponding to the Nef fusion proteins were excised from the gel and the incorporated radioactivity was counted. The extent of phosphorylation was three- to 10-fold greater, depending on the construct studied, with membrane extract than with the cytoplasmic extract (Fig. 3*b*).

Because previous reports indicated that Nef is phosphorylated by PKC, we checked to determine whether a PKC isoform was responsible for the Nef-associated kinase activity. GST–Nef was incubated with Jurkat cell extract and the kinase activity of the bead complexes was assayed in the absence (Fig. 4*a*, lane 1) or presence of the PKC inhibitors isoquinoline H7 (0·05 mM; Fig. 4*a*, lane 2) or bisindolylmaleimide GF 109203X (0·01 mM; Fig. 4*a*, lane 3). Both inhibitors significantly decreased the phosphorylation of Nef and two phosphorylated forms of GST–Nef were observed, suggesting that Nef is phosphorylated at more than one site (Fig. 4*a*, lanes 2 and 3). Inhibition experiments were repeated with GST–Nef and GST–Nef X/E in the absence or presence of GF 109203X (0·01 mM and 0·02 mM). GST–Nef (Fig. 4*b*), GST–Nef X/E (Fig. 4*c*) and p64 (Fig. 4*d*) were excised from the gel and the incorporated radioactivity was counted. Most of the kinase activity responsible for Nef phosphorylation was PKC-dependent. Neither EGTA (150 μM) nor Ca^{2+} (1 mM) changed the level of phosphorylation (data not shown). The

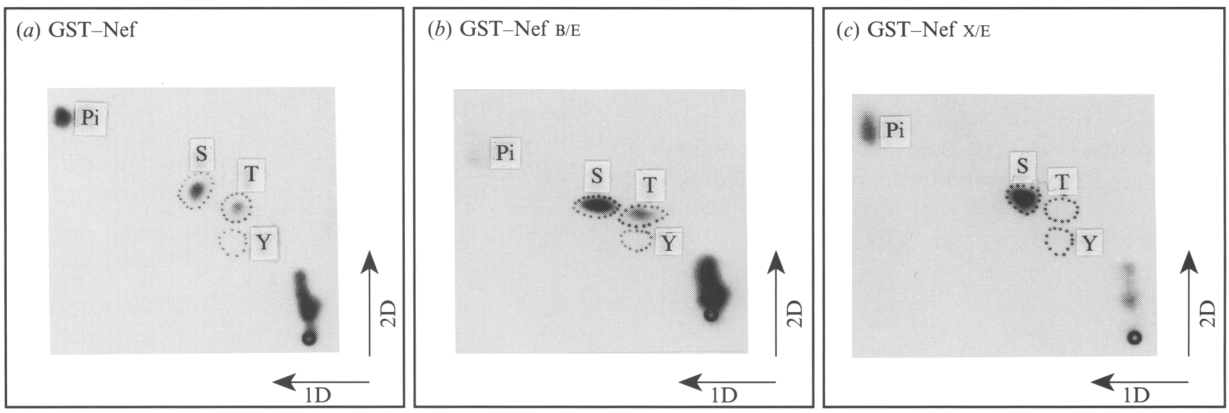

Fig. 5. Two-dimensional phosphoamino acid analysis of the phosphorylated GST–Nef and GST–Nef Δ mutants. The *in vitro* phosphorylated GST–Nef and GST–Nef Δ mutants were resolved by SDS–PAGE and transferred to a PVDF membrane. Bands corresponding to the labelled recombinant proteins were excised and hydrolysed. The resulting phosphoamino acids were analysed by two-dimensional electrophoresis and detected by autoradiography. The positions of the phosphoamino acids (S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine), free phosphate (Pi) and the origin (o) are marked.

phosphorylation of p64 was not significantly reduced by this PKC inhibitor.

Phosphorylation of GST–Nef on threonine and serine residues in vitro

The Nef residues phosphorylated in each of the GST–Nef proteins were identified by phosphoamino acid analysis (see Methods). Nef (Fig. 5*a*) and Nef B/E (Δ 87–206; Fig. 5*b*) were phosphorylated on both threonine and serine residues. Nef X/E (Δ 36–206) protein was mainly phosphorylated on serine residue(s) (Fig. 5*c*); after a longer exposure a weak phosphorylation of threonine was observed (data not shown). These results indicate that there are at least three phosphorylation sites on Nef: a minor site on Thr-15 (Nef X/E protein contains only this threonine residue) and two major sites, one on serine located in the 35 N-terminal aa and one on threonine, located between aa 36 and aa 86. No phosphorylation of tyrosine residues was detected, even after a longer exposure.

Discussion

Nef was produced from the HIV-1_{LAI} isolate as a GST–Nef protein in the pGEX-2T vector. Neither purified recombinant GST–Nef nor the Nef released from the GST carrier had any autokinase activity, in agreement with the findings of others (Kaminchik *et al.*, 1990; Matsuura *et al.*, 1991; Harris *et al.*, 1992). The autophosphorylation activity described earlier (Guy *et al.*, 1987; Nebrada *et al.*, 1991) is now generally attributed to bacterial contamination (Backer *et al.*, 1991). *In vitro* phosphorylation assays performed with immobilized GST–Nef proteins that had been incubated with Jurkat cell lysate showed that GST–Nef and GST–Nef Δ mutants could bind cellular kinase(s) and be phosphorylated. The kinase responsible was physically associated with Nef and strongly inhibited by two PKC inhibitors, whereas EGTA and Ca^{2+} had no effect on phosphorylation. These results suggest that the kinase which binds to and phosphorylates Nef is a Ca^{2+}-independent PKC isoform. Phosphoamino acid analyses showed that only serine and threonine were phosphorylated. Three *in vitro* phosphorylation sites were found on Nef, within an 86 aa N-terminal fragment; however, our data cannot rule out the possibility that further phosphorylation sites exist in the C-terminal part of the molecule between aa 87 and aa 206. All these phosphorylations were PKC-dependent. The first site is Thr-15, which has already been described as a PKC phosphorylation site (Guy *et al.*, 1987, 1990). The patterns of phosphorylation of each Nef construct were compared in order to identify the two other potential phosphorylation sites out of four serine residues (at positions 6, 8, 9 or 34) and five threonine residues (at positions 44, 48, 51, 71 or 80). *In vivo* phosphorylation of Nef has been reported for Thr-15 and for other unidentified serine and/or threonine residues (Guy *et al.*, 1987), which could be those described here. In addition we found a 64 kDa Nef-associated protein (p64) phosphorylated on serine and/or threonine by a PKC-independent mechanism. These observations indicate that a Nef-associated kinase activity phosphorylates a 64 kDa substrate. This p64 might be a cellular protein kinase that autophosphorylates or, alternatively, a bacterial contaminant that serves as a substrate of the kinase. Its molecular mass indicates that p64 is not any of the Nef-binding proteins detected *in vitro* by Poulin & Levy (1992) or Harris & Coates (1993), using a similar

GST approach. Sawai *et al.* (1994) studied the interaction of Nef with cellular kinases in eukaryotic cells and, surprisingly, saw no phosphorylation of Nef. But they did describe a non-PKC serine kinase associated with Nef and suggested that it could have a molecular mass of 62 kDa; this could correspond to the p64 described here.

The *in vitro* observations described here remain to be confirmed *in vivo* in Nef-transfected cells or in HIV-1-infected cells. However, several authors suggest that Nef can modulate cytoplasmic signalling and that Nef phosphorylation might play a role in such effects (Guy *et al.*, 1987; Niederman *et al.*, 1992, 1993; Skowronski *et al.*, 1993; Bandres & Ratner, 1994; Bandres *et al.*, 1994). The physical interaction of Nef with cellular kinases and its phosphorylation described here might play a role and have significant effects on viral replication. Recent studies in our laboratory have also shown that the cell protein β-COP, an essential component of the molecular machinery for membrane traffic, can bind to Nef in HIV-1-infected cells (Benichou *et al.*, 1994). Since several potential phosphorylation sites were found in the β-COP sequence, including sites for PKC (Duden *et al.*, 1991), one can speculate that a multimolecular complex involving Nef, β-COP, protein kinases plus other as yet unidentified proteins is formed in HIV-1-infected cells. Further studies are needed to explore this possibility.

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