The human immunodeficiency virus type 1 Nef protein functions as a protein kinase C substrate in vitro

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The human immunodeficiency virus type 1 Nef protein was expressed in *Escherichia coli* as a C-terminal fusion with glutathione S-transferase (GST). The ability of GST–Nef to act as a substrate for cellular kinases *in vitro* was examined by incubation of purified GST–Nef fusion proteins, immobilized on glutathione–agarose beads, with cytoplasmic extracts from a number of human cell lines. In the presence of [γ-32P]ATP, phosphorylation of Nef occurred predominantly on serine residues. Studies with protein kinase inhibitors suggested that protein kinase C (PKC) was involved in Nef phosphorylation. This was supported further by the demonstration that purified PKC was also able to phosphorylate Nef in the absence of cell extract. Serine/threonine phosphorylation of Nef was also observed *in vivo* when Nef was expressed with a C-terminal GST or 6-histidine tag in *Spodoptera frugiperda* insect cells by recombinant baculoviruses. In extracts from Jurkat T cells and U937 monocyte/macrophages Nef also associated with a 57 kDa cellular protein that was itself phosphorylated *in vitro*. Phosphorylation of this Nef-associated protein was inhibited by heparin and is thus likely to be mediated by casein kinase II. The observation that PKC can phosphorylate Nef *in vitro* raises the possibility that PKC might play a role in regulating both Nef function and the physical interactions between Nef and cellular components.

Introduction

The *nef* gene is conserved throughout the primate lentiviruses and encodes a protein product of between 205 [human immunodeficiency virus type 1 (HIV-1)] and 265 [simian immunodeficiency virus (SIV)] amino acids that is co-translationally modified by the addition of an N-terminal myristic acid residue (Allan et al., 1985). Data from the SIV system have demonstrated that *nef* plays an important role in the infectious process *in vivo*: SIV mutants with deletions in the *nef* gene failed to generate a pathogenic infection and virus loads were 1000-fold lower than with wild-type virus (Kestler et al., 1991). The mechanism underpinning this role for *nef* in *vivo* is not clear but clues have emerged from a number of *in vitro* studies over the last few years. Firstly, Nef expression has been shown to result in the species- and cell type-independent down-modulation of the cell surface glycoprotein CD4 (Guy et al., 1987; Garcia & Miller, 1991; Garcia et al., 1993; Aiken et al., 1994; Anderson et al., 1994). This down-modulation requires the cytoplasmic domain of CD4, in particular a dileucine motif known to be required for endocytosis of a number of cell-surface receptors (Aiken et al., 1994). However, down-modulation is independent of serine phosphorylation within the cytoplasmic domain and thus occurs via a novel mechanism (Garcia et al., 1993). Secondly, Nef expression results in an enhancement in the infectivity of virions in a CD4-independent fashion and has been shown to be important for the replication of HIV-1 in primary, quiescent lymphocytes (Miller et al., 1994; Spina et al., 1994). Lastly, it has been shown that Nef can modulate signal transduction through the T cell receptor, in particular the activation of transcription factors NF-xB and AP-1 (Luria et al., 1991; Niederman et al., 1992, 1993; Bandres & Ratner, 1994). Any or all of these aspects of Nef function may be expected to contribute to its importance for efficient viral replication *in vivo*.

The biochemical details underlying Nef function remain to be elucidated. It was originally suggested that Nef had limited sequence homology to the nucleotide-binding sites of protein kinases (Samuel et al., 1987) and G proteins such as Ras (Guy et al., 1987) and further to this, that purified bacterially expressed Nef could bind and hydrolyse GTP and also autophosphorylate (Guy et al., 1987). We and others have subsequently shown that the observed nucleotide-binding activity of Nef was most likely due to the presence of bacterial GTP-binding proteins in the partially purified Nef preparations (Harris et al., 1992a; Backer et al., 1991; Nebreda et al., 1991),

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protein could act as a kinase substrate in vivo and further show that one of the kinases acting on Nef is PKC. However, contrary to previous reports (Guy et al., 1987; Laurent et al., 1990), phosphorylation of Nef by PKC in vitro occurred predominantly on serine residues and did not involve Thr-15. We also report that Nef is phosphorylated when expressed in insect cells by recombinant baculoviruses.

### Methods

**Construction of plasmids and purification of GST fusion proteins.** The construction of pGEX-Nef expression vectors and the purification of GST, GST-BH10 and GST-PCR2 fusion proteins have all been described previously (Smith & Johnson, 1988; Harris et al., 1992a). The construction of baculovirus vectors expressing Nef-GST and Nef-6H has also been described previously (Harris & Coates, 1993).

**Preparation of cellular extracts.** Monolayers of HeLa and U373-MG cells were trypsinized and washed twice in ice-cold PBS. Suspension cells Jurkat and U937 were collected by centrifugation and washed as above. The cells were then lysed by sonication (3 x 10 s) on ice at a concentration of 10^6 cells/ml in lysis buffer (20 mM-HEPES/NaOH pH 7.5, 1 mM-EGTA, 10 mM-sodium orthovanadate, 10 mM-sodium fluoride, 0.4 mM-PMSF, 1 mM-DTT with the addition of benzamide, leupeptin and pepstatin A to a final concentration of 5 μg/ml). Cell lysates were clarified by centrifugation (14000 r.p.m./30 min/4 °C), glycerol was added to 50% (v/v) and the lysates were stored at −20 °C.

**In vitro phosphorylation assay.** GST or GST-Nef fusion proteins were bound to glutathione-agarose (GA) beads at a concentration of 2 mg/ml of packed beads. Twenty μg of bound protein was used per reaction. The beads were washed once in 20 mM-HEPES/NaOH pH 7.5 before the addition of 25 μl of reaction mixture (20 mM-HEPES/NaOH pH 7.5, 1 mM-EGTA, 10 mM-μg/ml) plus 50 μM-ATP or GTP including 30 μCi [γ-32P]ATP (ICN; 7000 Ci/mmol) or 30 μCi [γ-32P]GTP (Amersham; > 5000 Ci/mmol), respectively. The reaction was then started by the addition of 25 μl of pre-cleared cell extract and incubated at 37 °C for 15 min. Unless otherwise indicated, all reactions were performed using Jurkat extracts. In the assay using purified PKC, 1 μl/0.09 U of enzyme (rat brain; Calbiochem) was added to the appropriate tubes with a further 25 μl of lysis buffer and incubated as above. Inhibitors were added as described in the figure legends. The beads were then washed five times with 0.5 ml 20 mM-HEPES/NaOH pH 7.5/1% Triton X-100 and boiled in reducing sample buffer before resolution by electrophoresis on a 12% SDS–PAGE gel. The proteins were transferred on to PVDF membrane (Immobilon-P; Millipore) by a standard electrotransfer method and either stained with Coomassie Brilliant Blue R250 or immunoblotted with monoclonal antibodies against Nef or GST as previously described (Harris & Coates, 1993), before autoradiography to visualize labelled phosphorylated products.

**Thrombin cleavage of GST–Nef fusion proteins.** In vitro phosphorylation was carried out as above. After the last wash, 30 μl of thrombin cleavage buffer (150 mM-NaCl, 2.5 mM-CalC14, 10 mM-Tris–HCl pH 8.0) was added to the beads together with 0.5 U of thrombin (Sigma). Reactions were incubated for a further 30 min at 37 °C and processed as described above.

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Fig. 1. Nef is phosphorylated in vitro by cellular protein kinases. Purified GST (lane 1), or GST–Nef (BH10 strain) (lanes 2 to 5) were immobilized on glutathione-agarose beads and incubated with cytosolic extracts from Jurkat (lane 2), HeLa (lane 3), U937 (lane 4) or U373-MG (lane 5) cells.
**PKC phosphorylation of HIV-1 Nef in vitro**

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Coomassie a-Nef a-GST

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Fig. 2. GST-Nef is phosphorylated on Nef amino acid sequences. In vitro-phosphorylated GST-Nef was incubated either with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) thrombin for 30 min at 37°C. After SDS-PAGE and transfer to PVDF, identical strips were stained with Coomassie Brilliant Blue R-250 (lanes 1 and 2), or immunoblotted with monoclonal antibodies to the N-terminus of Nef (lanes 3 and 4) or GST (lanes 5 and 6). Filters were then autoradiographed, the right hand panel shows an autoradiograph of the anti-GST panel (lanes 7 and 8).

**Phosphoamino acid analysis.** In vitro phosphorylation was carried out as above with the exception that the amount of \([\gamma^{32}P]ATP\) was increased to 50 \(\mu\)Ci/reaction. Multiple reactions were resolved by SDS-PAGE, transferred to PVDF membrane and stained with Coomassie Brilliant Blue R250 before autoradiography. Phosphorylated GST-Nef bands were cut from the membrane and the proteins hydrolysed by incubation at 110°C in 57 M-HCl for 1 h. Phosphoserine, phosphothreonine, and phosphotyrosine standards (Sigma; 0.5 \(\mu\)g of each) were added to the hydrolysate prior to separation by two-dimensional thin layer electrophoresis on cellulose plates (Kodak). Unlabelled standards were located by spraying the dried plate with 0.2% ninhydrin (Sigma) and the radioactive phosphorylated amino acids were visualized by autoradiography.

**In vivo phosphorylation of Nef.** Spodoptera frugiperda (SP9) insect cells were infected at 5 p.f.u./cell recombinant baculoviruses expressing Nef-GST (AcNef-GST), Nef-6H (AcNef6H) or \(\beta\)-galactosidase (AcRPlacZ) as negative control. At 24 h post-infection (p.i.) cells were incubated with 100 \(\mu\)Ci/ml \([\gamma^{32}P]orthophosphate\) (Amersham; PBS 11), and at 40 h p.i. were harvested by lysis in 10 mM-PIPES/NaOH pH 7.2, 120 mM-KCl, 30 mM-NaCl, 5 mM-MgCl\(_2\), 1% Triton X-100, 10% glycerol containing 10 mM-EDTA, 0.2 mM-PMSF, 2 \(\mu\)g/ml aprotinin, 1 \(\mu\)g/ml leupeptin and 10 \(\mu\)M sodium orthovanadate at a concentration of 2 x 10\(^6\) cells/ml. Lysates were incubated at 4°C for 30 min, clarified by centrifugation (12000 g for 5 min) and bound to either GA beads or Ni\(^{2+}\)-NTA agarose. After separation of bound proteins by SDS-PAGE and transfer on to PVDF membrane, \(^{32}P\)-labelled proteins were detected by autoradiography. The positions of Nef-GST and Nef-6H were confirmed by immunoblotting with a sheep anti-Nef polyclonal serum.

**Results**

**Cellular protein kinases phosphorylate HIV-1 Nef on serine residues in vitro**

The nef gene of HIV-1 strain BH10, in which the premature termination codon at residue 124 had been repaired by site-directed mutagenesis, was expressed as a C-terminal fusion with GST in E. coli and purified to homogeneity by affinity chromatography on GA beads as described (Harris et al., 1992a). To investigate whether Nef could be phosphorylated in vitro, purified GST–Nef was immobilized on GA beads and incubated with cytoplasmic extracts from a number of different human cell lines. The beads were washed extensively and phosphorylation of GST–Nef examined by SDS-PAGE and autoradiography. Fig. 1 shows that a major phosphorylated band of 55 kDa corresponding to GST–Nef was generated by kinases from Jurkat T cells, HeLa, U937 monocyte/macrophages and U373-MG astroglial cells (lanes 2 to 5). A number of degradation products were also phosphorylated. As these degradation products bound to GA beads they presumably contained intact GST and therefore represent C-terminal degradation of Nef sequences. This notion is confirmed by the fact that the majority of these degradation products reacted with a monoclonal antibody directed to the N-terminal six amino acids of Nef (see Fig. 2). Phosphorylation of these bands suggests that a site, or sites, of phosphorylation lies towards the N-terminus of Nef. An additional labelled band of apparent molecular mass 57 kDa was observed in the presence of extracts from Jurkat or U937 cells. This band may correspond to a cellular protein that associates with Nef or a phosphorylation event that alters the mobility of GST–Nef. The latter possibility is unlikely as this band failed to appear on immunoblots using a number of different anti-Nef sera (data not shown).

Although GST was not phosphorylated in the presence
of Jurkat cell extract (Fig. 1, lane 1) it was important to confirm that when GST–Nef was used as substrate only Nef amino acid sequences were phosphorylated. To accomplish this we exploited the thrombin cleavage site that links GST and Nef in the pGEX construct. In vitro phosphorylated GST–Nef was cleaved with thrombin and aliquots of cleavage products were analysed by SDS–PAGE followed by Coomassie Blue staining, immunoblotting with anti-Nef and anti-GST monoclonal antibodies to identify the Nef and GST moieties, and autoradiography to identify the phosphorylated product (Fig. 2). As expected this analysis demonstrated that only the Nef portion of the fusion protein was phosphorylated.

Phosphorylation of GST–Nef was shown to be alkali labile and acid resistant (data not shown) suggesting that both the 55 kDa and 57 kDa bands were serine/threonine rather than tyrosine phosphorylated. Further, to identify whether GST–Nef was phosphorylated in vitro on either serine or threonine residues, phosphoamino acid analysis was carried out: GST–Nef was phosphorylated in vitro and the reaction products separated by SDS–PAGE. The phosphorylated 55 kDa band was excised and subjected to acid hydrolysis followed by two-dimensional electrophoresis (Fig. 3). This analysis revealed that the predominant phospho-

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\text{Fig. 3. Phosphoamino acid analysis of in vitro phosphorylated GST–Nef. In vitro phosphorylated GST–Nef was hydrolysed by treatment with 5-7 m-HCl at 110 °C and amino acids separated by two-dimensional electrophoresis.}
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amino acid was phosphoserine, although a very low level of phosphothreonine was also observed.

**Nef is phosphorylated in vitro by PKC**

Having established that Nef could function as a kinase substrate in vitro we wished to identify the kinase(s) responsible. A possible candidate was PKC, since it had been previously shown that PKC could phosphorylate Nef both in vivo and in vitro (Guy et al., 1987). As PKC can only utilize ATP as phosphate donor, we examined whether the phosphorylation reaction showed any specificity for donor phosphate. In vitro phosphorylation reactions were carried with Jurkat extract in the presence of \([\gamma-^{32}\text{P}]\text{ATP}\) (left hand panel) or \([\gamma-^{32}\text{P}]\text{GTP}\) (right hand panel).
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Fig. 5. Inhibition of Nef phosphorylation by protein kinase inhibitors. In vitro phosphorylation assays were performed as described with the addition of heparin (10 μM, lane 3), a pseudosubstrate peptide inhibitor of cAMP-dependent kinases (PKI peptide, 2 μM; lane 4) a pseudosubstrate PKC inhibitor peptide (PKC peptide, 10 μM; lane 5), or 2 mM-CaCl₂ (lane 6).

we also included a primary clinical isolate of Nef (PCR2) (Harris et al., 1992a) to verify that phosphorylation of Nef was not allele restricted. PCR2 and BH10 were phosphorylated with equal efficiency although only the 55 kDa band was observed in the presence of PCR2. Fig. 4 shows that both nucleotides were utilized with equal efficiency, implying that PKC was not involved. However, it was conceivable that PKC phosphorylation of Nef in the presence of [γ-³²P]GTP could have been permitted by ADP transphosphorylation by enzymes present in cell extracts. In vitro phosphorylation reactions were therefore carried out in the presence of a number of different protein kinase inhibitors (Fig. 5). Phosphorylation of the 55 kDa GST–Nef band was unaffected by heparin (an inhibitor of casein kinase II, lane 3), or a peptide inhibitor of cAMP-dependent kinases (lane 4). However phosphorylation was strongly inhibited by a pseudosubstrate peptide inhibitor of PKC (lane 5). We therefore tested whether purified PKC could phosphorylate Nef (Fig. 6). Commercially available PKC (purified from rat brain) was able to efficiently phosphorylate Nef (lane 4). Phosphorylation of Nef by both purified PKC and Jurkat extract was inhibited by two specific PKC inhibitors with distinct modes of action: the pseudosubstrate peptide and bisindolylmaleimide (BIM), a competitive inhibitor of the ATP-binding site. As expected, purified PKC showed an absolute requirement for ATP as phosphate donor (data not shown). In contrast, phosphorylation of the 57 kDa band was inhibited by heparin but was not significantly affected by inhibitors of PKC or cAMP-dependent kinases (Fig. 5), suggesting that this phosphorylation product was the result of casein kinase II activity. This suggestion was confirmed by the absence of the 57 kDa band from GST–Nef preparations phosphorylated by purified PKC (Fig. 6). PKC isoenzymes can be grouped into two categories based on their requirements for Ca²⁺ for activation (Osada et al., 1990): isotypes α, β, β₂ and γ require Ca²⁺, whereas δ, ε and ζ are Ca²⁺-independent due to the lack of the C2 calcium-binding domain. The presence of 1 mM-EGTA in the cell extracts suggested that this latter group of Ca²⁺-independent PKC isotypes were responsible for Nef phosphorylation. This notion was backed up by the observation that addition of 2 mM-CaCl₂ to the reactions containing cell extracts did not enhance phosphorylation (Fig. 5, lane 6).
In vivo phosphorylation of Nef

We were unable to detect in vivo phosphorylation of Nef when expressed in mammalian cells, possibly because only low levels of Nef expression can be attained without cytotoxicity. However, we had previously demonstrated that the baculovirus expression system could support high levels of Nef expression (Harris & Coates, 1993; Harris & Neil, 1994). This system was therefore exploited to investigate the possibility that Nef might be phosphorylated in vivo. Sf9 cells infected with recombinant baculoviruses expressing Nef with either GST (Nef−GST) or six histidines (Nef−6H) at the C terminus were labelled with [32P]orthophosphate. The resulting fusion proteins (Nef−GST or Nef−6H) were purified from lysates by affinity chromatography on GA beads or Ni²⁺−NTA agarose, respectively, and analysed by SDS-PAGE and autoradiography (Fig. 7). This result clearly demonstrates that both Nef−GST and Nef−6H were phosphorylated when expressed in Sf9 cells. Furthermore the phosphorylated bands were alkali sensitive, indicating that phosphorylation was occurring on serine or threonine residues (data not shown).

Discussion

In this report we demonstrate that the HIV-1 Nef protein is able to function as a kinase substrate in vitro. PKC was preliminarily identified as one of the kinases capable of phosphorylating Nef by virtue of the fact that phosphorylation of Nef by cell extracts was inhibited by specific PKC inhibitors, and furthermore, purified PKC could phosphorylate Nef in the absence of cell extract.

Fig. 7. In vivo phosphorylation of Nef in recombinant baculovirus infected Sf9 cells. Sf9 cells were infected with recombinant baculoviruses as indicated at 5 p.f.u./cell and labelled from 24–40 h p.i. with [32P]orthophosphate. Affinity purified Nef−GST or Nef−6H were processed by SDS-PAGE and transferred to PVDF membrane followed by autoradiography. Parallel samples were analysed by immunoblotting with a polyclonal sheep anti-Nef serum followed by donkey anti-sheep horseradish peroxidase conjugate (Sigma), and developed using the enhanced chemiluminescence system (ECL; Amersham). Lysates from cells infected with AcRPlacZ (expressing β-galactosidase) were processed identically and used as negative controls.
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(1987) that demonstrated PKC phosphorylation of Nef both in vivo, following infection of BHK-21 cells with recombinant vaccinia virus expressing Nef, and in vitro by incubation of purified bacterially expressed Nef with PKC. However, although that work did not directly demonstrate the identity of the phosphorylated amino acid(s), Thr-15 was implicated as an alanine substitution mutant was not phosphorylated in vivo in recombinant vaccinia virus-infected BHK-21 cells. In contrast an alanine was present at position 15 in the Nef protein used in this study (BH10) and phospho-amino acid analysis revealed that the predominant phosphorylated residue was serine (Fig. 3). This discrepancy may be explained by differences in the purification protocols. Guy et al. (1987) obtained 75% pure Nef protein by SDS solubilization of an insoluble fraction. In contrast, the protein used in this study was purified to homogeneity by affinity chromatography on glutathione agarose and was not exposed to denaturing agents. Our data therefore imply that in native Nef, Thr-15 is not the sole target for PKC action. Further experiments with a number of primary clinical isolates of Nef that contained threonine residues at position 15 (Harris et al., 1992a) indicated that the presence of this residue did not enhance the levels of phosphorylation (data not shown). Although PKC cannot utilize GTP as phosphate donor, phosphorylation of Nef in the presence of [γ-32P]GTP does not rule out PKC as the kinase since enzymes present in cell extracts may transphosphorylate ADP.

Amino acid sequencing of tryptic peptides derived from in vitro phosphorylated GST–Nef has thus far failed to identify the phosphorylated serine(s). HPLC and two-dimensional separations of tryptic digests revealed the existence of three phosphorylated species (data not shown), suggesting that up to three phosphorylation sites may exist. Mutation of the serine at position 6 did not affect the levels of phosphorylation (data not shown), however, there are a number of serine residues in Nef that lie in environments rich in basic amino acids that might be candidate PKC phosphorylation sites, these include serines at 103 and 169.

The identity of the 57 kDa phosphorylated band is unknown. It is likely to be a cellular protein that interacts with Nef as it was not detected by immunoblotting with anti-Nef sera (data not shown). We had previously identified proteins of 55/57 kDa in extracts of metabolically labelled Jurkat cells that interacted with a baculovirus-expressed myristylated Nef–GST fusion protein in vitro (Harris & Coates, 1993). These proteins were also observed to a lesser extent in association with non-myristylated Nef–GST (Harris & Coates, 1993) and E. coli-expressed GST–Nef (Harris et al., 1992b). It is tempting to speculate that the phosphorylated 57 kDa band observed in this study corresponds to the metabolically labelled 57 kDa protein identified in the previous study. In the light of our recent observation of a direct interaction between Nef–GST and CD4 in vivo (Harris & Neil, 1994) it is interesting to note that the extracts that gave rise to the 57 kDa band were derived from CD4-positive cells. However CD4 was not detected in GST–Nef precipitates by immunoblotting (data not shown), thus the 57 kDa band is unlikely to correspond to CD4. Furthermore, CD4 is known to be serine phosphorylated by PKC (Shin et al., 1994), whereas phosphorylation of the 57 kDa band described here was inhibited by heparin, implicating casein kinase II. Interestingly, a recent report (Sawai et al., 1994) described an as yet unidentified 62 kDa protein (62K) from Jurkat T cells that co-immunoprecipitated with a CD8–Nef fusion protein and was serine phosphorylated when immunoprecipitates were subjected to in vitro kinase assays. The position of the 62K protein on gels is very similar to that observed for the 57K protein, differences in gel systems may account for the discrepancy in observed molecular weights. Further indirect evidence that these proteins may be related lies in the fact that their phosphorylation was unaffected by inhibitors of either PKC or PKA.

As yet we have not been able to demonstrate serine phosphorylation of Nef in vivo in mammalian cells constitutively expressing Nef. This may be explained by the apparent cytotoxicity of Nef and the consequence that only low levels of Nef expression can be tolerated by mammalian cells. In addition, in vivo phosphorylation of Nef may be a transient event, only occurring under specific conditions of cell activation. Experiments are presently underway to develop inducible mammalian expression systems that may help to clarify this discrepancy. However, we have observed serine/threonine phosphorylation of Nef expressed in Sf9 insect cells by recombinant baculovirus vectors with either a C-terminal GST or six histidine tag (Fig. 7), demonstrating that Nef can be phosphorylated in vivo, albeit in a heterologous system.

The role of phosphorylation in the function of Nef in vivo remains to be elucidated. Phosphorylation plays a critical role in modulating the activity of a large number of cellular and viral proteins. In addition, phosphorylation is involved in protein–protein interactions and can influence the subcellular localization of proteins. A pertinent example of these two roles of phosphorylation is the myristylated alanine-rich C kinase substrate protein (MARCKS), a protein involved in regulating actin/membrane interactions (Adlerem, 1992). PKC phosphorylation of MARCKS results in its release from the plasma membrane and inhibits its binding to...
calmodulin and actin. The similarities between Nef and MARCKS (they are both PKC-phosphorylated, myristylated proteins located on the plasma membrane) lead to the intriguing possibility that PKC phosphorylation might regulate both the localization of Nef on the plasma membrane and the interactions between Nef and cellular proteins. Amongst its many functions PKC plays a pivotal role in the process of T cell activation (Crabtree, 1989). Given the previously documented effects of Nef on signal transduction (Luria et al., 1991; Niederman et al., 1992, 1993; Bandres & Ratner, 1994) we suggest that PKC phosphorylation of Nef may have important functional consequences for the life cycle of HIV-1.

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