Protein Kinase C-mediated Phosphorylation of HIV-I Nef in Human Cell Lines*

(Received for publication, December 6, 1996, and in revised form, February 6, 1997)

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Stable human cell lines expressing the human immunodeficiency virus type I (HIV-I) Nef protein from inducible promoters were used to analyze the phosphorylation status of Nef in vivo. Nef phosphorylation in both HeLa and Jurkat cells was stimulated by phorbol ester treatment. Phosphoamino acid analysis revealed a predominance of phosphoserine with a small proportion of phosphothreonine. Treatment of cells with selective protein kinase inhibitors revealed that Nef phosphorylation was markedly reduced by bisindolylmaleimide, an inhibitor of protein kinase C, but was unaffected by inhibitors of mitogen-activated protein kinase kinase or cAMP-dependent kinase. These data implicate protein kinase C in Nef phosphorylation in vivo, and thus confirm and extend earlier in vitro data. Phosphorylation of a nonmyristoylated Nef mutant was impaired, suggesting that membrane targeting of Nef was required for phosphorylation. This was expected given that activated protein kinase C translocates from the cytosol to the plasma membrane. However, analysis of the subcellular localization of phosphorylated wild-type Nef revealed that both the cytosolic and membrane-associated pools of Nef were phosphorylated to an equivalent extent. Thus the significance of myristoylation for Nef function may be in influencing protein conformation, although these data could be explained by a transient and dynamic interaction between myristoylated Nef and the plasma membrane.

The HIV-I Nef protein is a 206-amino acid polypeptide co-translationally modified at the N terminus by the addition of a myristate residue (1). This modification is, at least in part, responsible for the association of a proportion of Nef with cellular membranes (2, 3). Nef has been shown to have a number of biological functions in vitro that may be pertinent to its role in disease. In particular the presence of Nef in HIV-I-infected cells enhances the production of infectious virions (4, 5), and this has been recently shown to involve the incorporation of Nef into the virus particle and its subsequent cleavage by the viral protease (6, 7). However the precise biochemical mechanisms underpinning the enhanced infectivity of Nef+ virions remains to be defined. Nef expression also results in the down-modulation of CD4 from the cell surface (8, 9), and this function is apparently independent of its role in enhancing viral infectivity (10). Nef-mediated down-modulation of CD4 results from increased rates of CD4 endocytosis and lysosomal targeting/degradation (11–13), although again the precise biochemical mechanisms remain obscure. Finally Nef has been reported to have effects on signal transduction pathways both in lymphocytes and other cell types (14–17). In this context effects of Nef on induction of interleukin-2 synthesis have been observed by some workers (18–20), but there is little agreement in this area as to the precise effects of Nef and the potential role of this aspect of Nef function in viral replication is currently unclear.

In an attempt to unravel the biochemical mechanisms of Nef function, a number of groups have analyzed the cellular proteins with which Nef interacts, both in vitro and in vivo. A significant proportion of these Nef-interacting proteins have been identified as protein kinases of both the tyrosine and serine/threonine families. A proline-rich motif (amino acids 70–79) has been shown to interact with the SH3 domains of the Src family tyrosine kinases Hck (21, 22) and Lck (20, 23, 24). In addition a peptide corresponding to this motif inhibited the in vitro binding of Nef to MAP kinase (20, 24). Binding of Nef to both Lck and MAP kinase has been shown to inhibit kinase activity (23, 24). Nef has also been shown to associate with a 65K serine/threonine kinase that has been shown to be a member of the p21-associated kinase family (25, 26), and recently an association between Nef and PKC θ has been reported (27). However, none of these associations has been shown to result in the phosphorylation of Nef by the interacting kinase. An early study demonstrated that Nef was phosphorylated in recombinant vaccinia virus-infected BHK21 cells and that this phosphorylation could be stimulated by treatment of cells with phorbol ester (8). This study also showed that partially purified bacterially expressed Nef could be phosphorylated in vitro by purified PKC. We (28) and others (29) have subsequently extended the latter observation, demonstrating that bacterially expressed glutathione S-transferase-Nef fusion proteins purified to homogeneity on glutathione-agarose beads could be phosphorylated on serine and threonine residues, both by purified PKC and PKC present in lysates from mammalian cells. We now demonstrate that Nef expressed in human HeLa and Jurkat cells is phosphorylated in vivo. This phosphorylation was stimulated by phorbol ester treatment of cells and was inhibited by a selective PKC inhibitor, but not by inhibitors of MAP kinase kinase or cAMP-dependent kinase. Phosphoryla-
tion of Nef did not demonstrate an absolute dependence on association of Nef with cytoplasmic membranes as a nonmyristoylated mutant was also phosphorylated, albeit with reduced efficiency. Additionally, phosphorylation did not affect the observed distribution of Nef between cytosolic and membrane fractions, as both populations of Nef were equally efficiently phosphorylated.

MATERIALS AND METHODS

Cell Lines and Constructs—The construction of HeLa and Jurkat cells expressing Nef from tetracycline-dependent metal-responsiveness promoters has been described previously (30). Construction of the nonmyristoylated Nef mutant has been described (31). Cells were maintained in DME (HeLa) or RPMI 1640 (Jurkat) containing 10% fetal calf serum, supplemented with 1 μg/ml tetracycline in the case of the HeLa cells to repress the tet-responsive promoter (32).

Chemicals—All chemicals and reagents were purchased from Sigma, with the exception of 4-(2-aminoethyl)benzenesulfonylfluoride-HCl (AEBSF), bisindolylmaleimide-HCl (BIM), and 2′-amino-3′-methoxyflavone (PD98059), which were purchased from Calbiochem. The myristoylated PKI inhibitor peptide was a kind gift from Roger Clegg (Hannah Research Institute, Ayr, Scotland).

Metabolic Labeling—For phosphorylation assays HeLa cells were seeded at 5 × 10⁵ cells/dish in the absence of tetracycline. After 40 h, monolayers were washed once in DME without sodium phosphate but supplemented with 1% dialyzed fetal calf serum. Cells were then incubated for 4 h at 37 °C in 2 ml of labeling medium containing 200 μCi/ml [35S]p-orthophosphate (Amersham Corp.) to equilib rate the intracellular ATP pools with labeled phosphate, prior to treatment with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 30 min. For experiments involving kinase inhibitors, the inhibitors were added at the same time as the label and were thus present throughout the labeling period. Monolayers were washed twice with ice-cold phosphate-buffered saline and harvested into 1 ml of phosphate-buffered saline containing 10 mM EDTA. Cell pellets were lysed in 500 μl of 10 mM PIPES-NaOH, pH 7.2, 0.5 mM MgCl₂, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 10% glycerol containing 100 μM okadac acid, 10 mM sodium fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.2 mM AEBSF for 30 min at 4 °C. Jurkat cells were treated similarly as described above but with 2.5 × 10⁷ cells for incubation with the presence of 100 μM ZnCl₂ prior to labeling. Unlabeled lysates (for immunoblotting) were processed in parallel.

Labeling with [35S]methionine was carried out for 4 h in DME without methionine but supplemented with 1% dialyzed fetal calf serum and 100 μCi/ml [35S]methionine (Tran 35S-label, Radiochemical Centre, Amersham, UK). Labeling with ³²P-histidine labeled myristic acid was carried out for 16 h in complete DME supplemented with 1% dialyzed fetal calf serum and 200 μCi/ml ³²P-labeled myristic acid (40–60 Ci/mmol, Amersham Corp.). Myristic acid (supplied in ethanol) was dried down under vacuum and resuspended in MesoSO to a final volume of 1% of the labeling medium.

Immunoprecipitation and Immunoblotting—Lysates were adjusted to 0.5 mM KCl and preclarified by the addition of 20 μl of protein G-Sepharose beads and incubation on a rotating platform for 2 h at 4 °C. Nef was immunoprecipitated overnight at 4 °C by the addition of a murine monoclonal antibody specific for the N-terminal 7 amino acids of Nef (3 μl of ascitic fluid). 10 μl of protein G-Sepharose beads were added (in 50 μl of lysis buffer) and incubated for a further 4 h at 4 °C on a rotating platform. Beads were washed three times in lysis buffer containing 0.5 mM KCl and once in lysis buffer prior to addition of 20 μl of 1× boiling buffer (0.8% SDS, 8% glycerol, 2% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.8), analysis by 15% SDS-PAGE, and autoradiography. For immunoblotting gels were transferred to polyvinylidene difluoride membrane (Millipore Immobilon P) using a Bio-Rad semidy electromoblotting apparatus. After blocking in TBS-T (25 mM Tris, 137 mM NaCl, 0.1% Tween-20) containing 10% (v/v) dried skimmed milk, membranes were sequentially probed with a sheep polyclonal Nef serum (1:10000) and donkey anti-sheep horseradish peroxidase (Sigma), prior to visualization by enhanced chemoluminescence (Amersham Corp.).

Regulable expression of Nef in Human HeLa and Jurkat Cell Lines—We have previously described the construction of stable cell lines expressing Nef from inducible promoters (30). Stable HeLa cell lines were generated using a derivative of the tetracycline responsive system originally developed by Gossen and Bujard (32) in which the components of the system were cloned into the pREP series of episomal vectors. This system proved to be unsuitable for regulatable Nef expression in Jurkat cells as expression was constitutive, so in these cells Nef was expressed from a modified metallothionein promoter (33). Fig. 1 demonstrates the inducibility of Nef expression in these cells. HeLa cells were incubated with and without tetracycline (1 μg/ml), and Jurkat cells were treated with 100 μM ZnCl₂ for 24 h prior to harvesting and analysis of Nef expression by immunoblotting. In both cases Nef expression can be induced at least 10-fold. In all the phosphorylation experiments described subsequently Nef expression was induced either by growth in the absence of tetracycline (HeLa cells) or in the presence of 100 μM ZnCl₂ (Jurkat cells).

Phosphorylation of Nef in Human HeLa or Jurkat T Cells—Phosphorylation was determined by incubation with [³²P]p-orthophosphate and lysates were immunoprecipitated with a Nef-specific murine monoclonal antibody. Fig. 2 demonstrates that in HeLa cell lysates a 28-kDa phosphorylated band corresponding to Nef was immunoprecipitated by the Nef monoclonal antibody from cells expressing Nef but not from control cells stably transfected with an empty vector. Phosphorylation of this species was stimulated at least 8-fold (as judged by guest on July 25, 2018http://www.jbc.org/Downloaded from
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Inhibition of Nef Phosphorylation by a Selective Inhibitor of PKC—As PKC is known to translocate from the cytosol to the plasma membrane once activated by phorbol ester binding, it was conceivable that phosphorylation would require membrane localization of Nef. Myristoylation is absolutely required for the stable association of Nef with a cytoplasmic membrane fraction, therefore HeLa and Jurkat cells expressing a nonmyristoylated Nef mutant (Gly2 \( \rightarrow \) Ser) were utilized to address this question. To confirm that this mutant was indeed nonmyristoylated, HeLa cells were metabolically labeled with either \([^{3}H]\)myristate or \([^{35}S]\)methionine and immunoprecipitated using the N-terminal-specific Nef monoclonal antibody. Fig. 5a shows that this Nef mutant could be labeled with \([^{35}S]\)methionine, but failed to incorporate \([^{3}H]\)myristate. In contrast wild-type Nef was myristoylated as demonstrated by the incorporation of \([^{3}H]\)myristate. The intracellular localization of the wild-type and nonmyristoylated Nef proteins was analyzed by subcellular fractionation of HeLa cells expressing each protein. Cytosolic and membrane-associated fractions of these cells were analyzed by immunoblotting with a Nef specific sheep polyclonal serum (Fig. 5b). The data confirm that a proportion of wild-type Nef molecules was stably associated with the membrane fraction; however, it is clear that more than 50% of myristoylated Nef molecules remained cytosolic. In comparison the nonmyristoylated Nef mutant failed to associate with the distinct kinase that subsequently phosphorylates Nef. A number of other lines of evidence point to PKC as a direct effector of Nef phosphorylation; in vitro data from a number of laboratories has indicated that purified PKC can phosphorylate purified Nef (8, 28, 29), and in addition our previous data demonstrated that only kinase inhibitors selective for PKC suppressed Nef phosphorylation in vitro by kinases present in cell extracts (28). To investigate whether PKC plays a role in Nef phosphorylation in vivo HeLa and Jurkat cells expressing Nef were treated with a number of selective kinase inhibitors, prior to PMA stimulation and analysis of Nef phosphorylation. As shown in Fig. 4 treatment of cells with a cell-soluble inhibitor of PKC, BIM, dramatically reduced the levels of PMA-induced Nef phosphorylation in comparison to the level of phosphorylation detectable in the absence of inhibitor. Neither PD98059, a synthetic inhibitor of MAP kinase kinase (35), or a myristoylated peptide inhibitor of cAMP-dependent kinase, corresponding to residues 5–24 of the heat-stable inhibitor PKIα (36) had any significant effect. These data confirm the relevance of our previous in vitro data and attest to a role for PKC in Nef phosphorylation in vivo.
membrane fraction and was exclusively cytosolic. Identical results were obtained for expression and localization of the wild-type and nonmyristoylated Nef mutant in Jurkat cells (data not shown).

HeLa and Jurkat cells expressing wild-type and nonmyristoylated Nef were then labeled with [32P]orthophosphate and Nef phosphorylation was analyzed by immunoprecipitation. Fig. 6a demonstrates that a phosphorylated species corresponding to the nonmyristoylated Nef mutant could be detected; however, in comparison with wild-type Nef the extent of phosphorylation was clearly greatly reduced. Unlabeled extracts immunoprecipitated in parallel and analyzed by immunoblotting revealed that comparable amounts of the two Nef species were present in immunoprecipitates (Fig. 6b), although it should be noted that in the HeLa cell lines the nonmyristoylated mutant was expressed at a lower level than the wild-type (see Fig. 5a). Thus it is clear that, although phosphorylation does not demonstrate an absolute requirement for myristoylation, the latter modification greatly enhances the ability of Nef to function as a phosphorylation substrate.

It has been reported that the distribution of some myristoylated proteins such as MARCKS (37) and HIV-I p17Gag (38) is regulated by phosphorylation. These proteins are stably anchored into the plasma membrane by a combination of myristoylation and electrostatic interactions between basic amino acids and acidic phospholipids. Phosphorylation results in their dissociation from the membrane by electrostatic repulsion. To investigate whether this situation might also apply to Nef, HeLa cells expressing wild-type Nef were labeled with either [32P]orthophosphate or [35S]methionine and fractionated into cytosolic (Cyt.) and membrane-associated (Mem.) fractions by hypotonic lysis, and the distribution of Nef was analyzed by immunoblotting of fractions with a sheep polyclonal anti-Nef serum.

**Fig. 5.** Nonmyristoylated Nef fails to associate with cytoplasmic membranes. a, stable HeLa cell lines expressing either wild-type Nef (wt), or a point mutation changing residue glycine 2 to serine (G2S), were metabolically labeled with either [35S]methionine or 3H-labeled myristic acid prior to immunoprecipitation and visualization by SDS-PAGE and fluorography. b, HeLa cells expressing wild-type Nef or the G2S mutant were fractionated into cytosolic (Cyt.) and membrane-associated (Mem.) fractions by hypotonic lysis, and the distribution of Nef was analyzed by immunoblotting with a sheep polyclonal anti-Nef serum.

**Fig. 6.** Phosphorylation of Nef is enhanced by myristoylation. a, HeLa or Jurkat cells stably transfected with either wild-type Nef (wt), the nonmyristoylated mutant (G2S), or an empty vector (Cont) were induced, labeled with [32P]orthophosphate, and stimulated with PMA as described under "Materials and Methods." Nef immunoprecipitates were analyzed by SDS-PAGE and autoradiography. b, unlabeled cells were processed in parallel to determine the overall levels of the two Nef proteins. Nef immunoprecipitates were analyzed by immunoblotting with a sheep polyclonal anti-Nef serum. The prominent higher molecular weight bands present in all five lanes is a result of cross reactivity of the secondary antibody to the heavy chain of the immunoprecipitating antibody.

**Fig. 4.** Inhibition of Nef phosphorylation by a specific PKC inhibitor. Induced HeLa-Nef (a) or Jurkat-Nef (b) cells were labeled with [32P]orthophosphate for 4 h in the presence of the following selective protein kinase inhibitors: BIM (10 μM), PD98059 (10 μM) (35), or a myristoylated peptide corresponding to residues 5–24 of the heat-stable inhibitor of cAMP dependent kinase (myrPKI: 100 μM) (36), prior to stimulation with PMA for 30 min. The first lane of each panel shows the results from untreated cells.

**DISCUSSION**

In this study we report that the HIV-I Nef protein can be phosphorylated in vivo by a cellular serine/threonine protein kinase, and we present evidence to suggest that this phosphorylation is mediated by members of the PKC family. The evidence for the latter statement is 2-fold. First, Nef phosphorylation is stimulated by treatment of Nef expressing cells with the phorbol ester PMA, a well characterized activator of PKC (Fig. 2a). Second, Nef phosphorylation was reduced by a selective PKC inhibitor, BIM, but not by inhibitors of cAMP-dependent kinase or MAP kinase kinase (Fig. 4). Although previous in vitro data both from our laboratory and others have indicated that purified PKC can directly phosphorylate recombinant Nef in the absence of other cellular factors (8, 28), it cannot be ruled out that in vivo PKC acts indirectly to induce Nef phosphorylation by activating a distinct protein kinase. In this regard it should be noted that BIM treatment did not completely abrogate phosphorylation of Nef, but merely reduced it to levels comparable to those observed in unstimulated cells (in the absence of PMA). One explanation for this observation is that the constitutive phosphorylation of Nef is mediated by a distinct kinase activity that is itself stimulated by PKC.

Activation of PKC by phorbol ester binding is concomitant...
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FIG. 7. Phosphorylation does not affect the subcellular localization of Nef. a, control or Nef expressing HeLa cells were labeled with [32P]orthophosphate as described and fractionated by hypotonic lysis prior to immunoprecipitation. b, HeLa-Nef cells were labeled with [35S]methionine, fractionated by hypotonic lysis, and immunoprecipitated in parallel to demonstrate the overall distribution of Nef molecules within the cell.

with its translocation from the cytosol to the plasma membrane where it inserts into the lipid bilayer (34). The majority of PKC with its translocation from the cytosol to the plasma membrane (30) encodes a protein functionally active in both HeLa and Jurkat cells, as judged by a number of criteria including enhancement of viral infectivity (30), CD4 down-modulation (30), and inhibition of AP-1 transcription factor induction. It is likely, therefore, that phosphorylation plays a role in the function of Nef. That role remains elusive; however, one immediate functional consequence is that phosphorylation will influence the interactions between Nef and cellular proteins. There are many pertinent examples of phosphorylation-dependent protein-protein interactions, for example MARCKS binding to calmodulin is inhibited by PKC phosphorylation (37), and in some instances phosphorylation has been implicated in phosphotyrosine-independent interactions with SH2 domains (48, 49). Further insights into the functional consequences of Nef phosphorylation must await the results of future experiments with mutated genes.

Acknowledgment—We thank Professor Jim Neil for critical reading of this manuscript.

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J. Biol. Chem. 1997, 272:12289-12294.
doi: 10.1074/jbc.272.19.12289

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