The Nef Protein of Human Immunodeficiency Virus Type 1 Enhances Serine Phosphorylation of the Viral Matrix

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The human immunodeficiency virus type 1 matrix (MA) protein is phosphorylated during virion maturation on its C-terminal tyrosine and on several serine residues. Whereas MA tyrosine phosphorylation facilitates viral nuclear import, the significance of MA serine phosphorylation remains unclear. Here, we report that MA serine but not tyrosine phosphorylation is strongly enhanced by Nef. Mutations that abrogated the membrane association of Nef and its ability to bind a cellular serine/threonine kinase greatly diminished the extent of virion MA serine phosphorylation. Correspondingly, a protein kinase coimmunoprecipitated with Nef could phosphorylate MA on serine in vitro, producing a phosphopeptide pattern reminiscent of that of virion MA. Recombinant p21-activated kinase hPAK65, a recently proposed relative of the Nef-associated kinase, achieved a comparable result. Taken together, these data suggest that MA is a target of the Nef-associated serine kinase.

Lentiviruses are distinguished among retroviridae by the presence of genes encoding accessory proteins that fulfill important functions in the control of viral replication (48, 49). The primate lentiviruses are further characterized by a unique open reading frame at the distal end of the viral genome, *nef*. The crucial role of Nef was initially revealed by studies in the simian immunodeficiency virus-rhesus macaque model of AIDS pathogenesis, where it was found to be essential for high levels of viral replication and disease progression in adult animals (25). More recently, *nef*-defective strains have been implicated in some cases of long-term survival to human immunodeficiency virus type 1 (HIV-1) infection in humans (11, 27).

The 206-amino-acid HIV-1 Nef protein is produced in abundance from the earliest stage of viral gene expression (26, 28). Myristoylated at its N terminus, Nef is predominantly cytoplasmic and associates with membranes and cytoskeletal elements (15, 24, 34). In tissue culture, Nef affects both the kinetics of virus replication and the biology of the host cell. Nef increases HIV-1 infectivity by acting at the stage of particle production to promote steps that immediately follow viral entry. This influence is particularly noticeable following the inoculation of quiescent primary lymphocytes and their subsequent activation (9, 33, 47). It reflects increased rates of viral DNA synthesis, apparently due to improved properties of the uncoated viral nucleoprotein complex rather than to a stimulation of the enzymatic activity of reverse transcriptase per se (2, 10, 44).

In parallel, Nef alters several cellular functions. First, it downregulates the surface expression of CD4 (22, 23) and of major histocompatibility complex class I molecules (45). This modulation occurs posttranscriptionally through mechanisms that include enhanced endocytosis (1, 31, 37, 43, 45). Nef also affects cellular activation pathways, particularly in T lymphoid cells (4, 35, 46). This may at least partly reflect its interaction with cellular protein kinases (38, 40), including a serine/threonine kinase that can be coimmunoprecipitated with Nef in a wide variety of cells (40). The membrane association of Nef, as

well as a critical arginine in its central domain, is required for recruiting this activity (41). Although the Nef-associated serine/threonine kinase has not yet been formally identified, recent experiments suggest that it belongs to the p21-activated kinase (PAK) family (29, 36, 42).

Matrix (MA) is the N-terminally myristoylated cleavage product of the Gag polyprotein precursor by the viral protease (12). An essential structural element of retroviruses, MA governs HIV-1 assembly by recruiting either directly or indirectly the other components of the virion at the plasma membrane. Mutations in HIV-1 MA can block assembly, prevent the incorporation of envelope into virions, or affect steps of the viral life cycle that immediately follow viral entry (7, 13, 14, 16–21, 30, 50, 51, 53–56). During particle formation, a small subset of HIV-1 MA undergoes phosphorylation on serine and tyrosine (6, 12, 18). Tyrosine phosphorylation occurs on the C-terminal residue of MA and triggers the binding of MA to integrase (18, 19). This allows for the recruitment of MA into the virion core and subsequently into the uncoated viral nucleoprotein complex (19). A nuclear localization signal in MA is then recognized by the cell nuclear import machinery, which directs the viral nucleoprotein complex to the nucleopore (7, 18–20, 51).

The present study was originated to examine the modalities of MA serine phosphorylation. We found that this modification, but not MA tyrosine phosphorylation, is strongly enhanced by Nef. In addition, mutations that abrogated the binding of Nef to a protein serine kinase diminished the extent of MA serine phosphorylation. Finally, MA could be an in vitro substrate for recombinant p21-activated kinase hPAK65, a proposed relative of the Nef-associated kinase.

MATERIALS AND METHODS

DNA constructions. The HIV-1_{HXB2}-derived R7 proviral construct was previously described (26); R9 (previously called R8 in reference 19) was obtained by cloning a *Bss*HII-*Bam*HI fragment, including the MA coding sequence, from HIV-1_{NL4.3} into R7. The Nef-defective clone ΔNef contains a frame-shift mutation at nucleotide 100 and the myristoylation-defective Nef_{G2A}, a glycine-to-alanine change at position 2 of the *nef* open reading frame (1). Nef_{RR106AA} was constructed by PCR-mediated mutagenesis, and its sequence was verified by dideoxy sequencing. Recombinant HIV-1_{HXB2} MA carrying an N-terminal histidine tag was produced in *Escherichia coli* by using the vector pET-15b (Novagen) and purified according to the manufacturer's instructions as previously described (18).

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Radiolabelling and biochemical procedures. CEM lymphoblastoma cells, obtained originally from Peter Nara, and SupT1 cells, obtained from James Hoxie through the NIH AIDS Research and Reference Reagent Program, were maintained in RPMI 1640 medium. Methods used for radiolabelling virions with [32 P]orthophosphate and for radioimmunoprecipitations, in vitro kinase assays, and Western blotting have been previously described (18). Phosphoamino acid and tryptic phosphopeptide analyses were performed as described by Boyle et al. (5). For the examination of the de novo phosphorylation of HIV MA in target cells, 5×10^6 CEM cells were radiolabelled for 24 h with 25 mCi of [32 P]H $_3$ PO $_4$ prior to infection with concentrated viruses (1 to 2 μ g of p24) in a small volume of complete RPMI 1640 (1 to 2 ml) containing 20 μ g of DEAE-Dextran/ml for 4 h. After three washes with phosphate-buffered saline to remove unadsorbed virions, cells were lysed in single detergent lysis buffer (39), and the nuclear pellet was extracted further with a high salt buffer (18). Both extracts were pooled and standardized by p24 enzyme-linked immunosorbent assay before immunoprecipitation with HIV-1 MA-specific antibodies.

Kinase assays with hPAK65 and PKC. Recombinant myc-tagged hPAK65 (1 to 2 μ g) (32) bound to protein G-Sepharose conjugated with monoclonal Myc antibody was washed once and incubated in 40 μ l of kinase buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM MgCl $_2$, 1 mM MnCl $_2$) containing 1 to 2 μ g of CDC42Hs previously loaded with GTP and 5 μ g of myelin basic protein (MBP) or recombinant MA. The reaction was initiated by adding 10 μ l of kinase buffer containing 50 μ M ATP and 5 μ Ci of [γ - 32 P]ATP and incubated for 20 min at 30°C. The reaction was stopped by adding 10 μ l of 5 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiling for 5 min. Samples were applied to an SDS-14% PAGE gel, transferred to a polyvinylidene difluoride membrane, stained with Coomassie blue, destained, dried, and exposed to X-ray film for 1 to 2 h. Phosphorylated bands were excised and subjected to phosphoamino acid and tryptic phosphopeptide analyses as previously described (5). MA phosphorylation mediated by protein kinase C (PKC) was similarly analyzed, using recombinant PKC (Upstate Biotechnology, Inc.) as the enzyme and histone H1 (Boehringer Mannheim) as a control substrate.

RESULTS

Virion MA serine but not tyrosine phosphorylation is enhanced by myristoylated Nef. The potential dependence of MA serine phosphorylation on other viral gene products was investigated. Virions purified from the supernatant of CEM cells infected with a *nef*-defective (Δ Nef) HIV-1 clone, or with one encoding a nonmyristoylated version of Nef (Nef $_{G2A}$), contained strikingly lower amounts of phosphorylated MA than wild-type virions (Fig. 1A). Of note, this difference was less apparent when examining MA molecules extracted from virus producer cells (data not shown). Tryptic peptide mapping studies revealed three major tryptic phosphopeptides of high chromatographic mobility in wild-type MA (Fig. 1B, left). These peptides contained exclusively phosphoserine, while phosphotyrosine was detected in minor phosphopeptides of low mobility, as previously described (data not shown) (18). MA molecules from *nef*-defective virions exhibited decreased amounts of all three of the major phosphoserine-specific peptides (Fig. 1B, right). In contrast, Nef did not influence the C-terminal tyrosine phosphorylation of MA (Fig. 1C), nor the phosphorylation of CA, another serine phosphoprotein of HIV-1 virions (data not shown). Multiple analyses performed on virions produced from either CEM or SupT1 T lymphoid cells confirmed that Nef stimulates the incorporation of phosphate at all the major serine phosphoacceptor sites of MA (data not shown). In contrast, no significant difference was noted in the levels of MA serine phosphorylation when viruses were obtained from the supernatant of transfected 293(T) human kidney cells (data not shown). Of note, the lack of Nef effect on MA tyrosine phosphorylation correlates with our previous observation that this modification can occur when MA is expressed in the absence of any other viral protein (18).

A Nef mutant that fails to associate with a cellular serine kinase exhibits decreased levels of MA serine phosphorylation. The membrane association of Nef is important for recruiting a cellular serine/threonine kinase, the binding of which also depends on an arginine residue at position 106 of HIV-1 Nef (29,

FIG. 1. Serine but not tyrosine phosphorylation of HIV-1 virion MA is stimulated by Nef. (A) Lysates of HIV-1 (R9) virions, expressing either wild-type (WT), truncated (Δ Nef), or nonmyristoylated (Nef $_{G2A}$) versions of Nef, produced in CEM cells in the presence of [32 P]orthophosphate were immunoprecipitated with anti-MA antibodies; products were analyzed by SDS-PAGE and autoradiography (left) or MA-specific Western blotting (right). In the Western blot, the high-molecular-weight band corresponds to the antibody light chain. (B) MA molecules from wild-type and Δ Nef virions were subjected to a tryptic phosphopeptide analysis. Unlabelled arrows point to major phosphoserine-containing peptides. Phosphotyrosine-specific peptides are poorly visible due to low intensity of signal. (C) Lysates of the indicated virions and infected CEM cells were subjected to Western blotting with anti-phosphotyrosine (left) or a mixture of MA- and CA-specific (right) antisera.

41, 42). We previously reported that a Nef mutant in which this and the upstream arginine are changed to alanine (Nef $_{RR106AA}$) is stably expressed and 50% as efficient as wild-type Nef for CD4 downregulation (3). As previously reported (29, 41, 42), this mutant was defective for the binding of a serine kinase activity (data not shown). The levels of virion MA serine phosphorylation were significantly decreased at all major serine phosphoacceptor sites in an HIV-1 derivative producing the Nef $_{RR106AA}$ protein (Fig. 2). In contrast, mutating the Nef arginine dipeptide did not affect CA phosphorylation (Fig. 2) or MA tyrosine phosphorylation (data not shown).

FIG. 2. A Nef variant defective for kinase binding fails to stimulate virion MA serine phosphorylation. Lysates of HIV-1 (R9) derivatives expressing wild-type (WT) or diarginine mutated ($RR106AA$) versions of Nef produced from [^{32}P]orthophosphate-labelled CEM cells were immunoprecipitated with anti-CA and anti-MA antisera; products were analyzed by SDS-PAGE and autoradiography (left) and by two-dimensional tryptic phosphopeptide mapping (right). The similar levels of phosphorylated CA in lanes corresponding to the wild-type and mutant viruses demonstrate that equal amounts of material were loaded.

Similar results were obtained when virions produced from Sup T1 cells were examined (data not shown). The association of Nef with a serine kinase thus appears to promote MA serine phosphorylation.

Postentry MA serine phosphorylation is Nef independent.

After virion internalization, MA is the substrate of additional phosphorylation events (6). When CEM cells labelled with orthophosphate were infected with HIV-1, a significant level of de novo phosphorylation of MA occurred within 4 h (Fig. 3A), whereas CA did not incorporate any phosphate (data not shown). MA molecules immunoprecipitated from CEM cells inoculated with either wild-type or Δ Nef viruses exhibited similar levels of de novo MA phosphorylation (Fig. 3A), indicating that Nef does not influence this process. Phosphoamino acid analyses revealed that in target cells phosphate was added only on serine residues (Fig. 3B). Finally, tryptic maps of MA molecules phosphorylated immediately after infection of CEM cells with either wild-type or *nef*-defective virions confirmed that Nef did not significantly affect this event (data not shown).

MA serine kinase activity can be coimmunoprecipitated with Nef. The above results incriminated the Nef-associated serine kinase as a potential mediator of MA phosphorylation. To explore this issue further, wild-type and myristoylation-defective versions of Nef were immunoprecipitated from cytoplasmic extracts of stably expressing CEM cells, and the resulting immune complexes were used in an in vitro kinase assay with or without recombinant MA as substrate. The products of the reaction were resolved by SDS-PAGE and autoradiography. As previously described (41), a 62-kDa phosphoprotein was observed in kinase reactions performed on immunoprecipitates obtained from cells producing wild-type but not non-myristoylated Nef (Fig. 4A, top). When recombinant MA was added to these reactions, phosphorylation of the Gag product

FIG. 3. MA serine phosphorylation in target cells is Nef independent. CEM cells incubated with [^{32}P]orthophosphate were acutely infected with wild-type (WT) and *nef*-defective (Δ Nef) R9 virions. MA molecules immunoprecipitated 4 h later were analyzed by SDS-PAGE and autoradiography (A) and by phosphoamino acid analysis (B). Only phosphoserine was detected, even after prolonged exposure. The data shown here are representative of two independent experiments.

was observed with wild-type Nef immunoprecipitates at levels that were significantly higher than those which arose nonspecifically with immunoprecipitates from both control and non-myristoylated Nef-expressing cells (Fig. 4A, middle). In this context, MA phosphorylation occurred only on serine residues (Fig. 4B) and produced a tryptic phosphopeptide map comparable to that observed with virion MA (Fig. 4C). Of note, the pattern obtained with recombinant MA, which was derived from HIV-1_{HXB2}, differed slightly from that yielded by MA molecules purified from R9 virions, derived from HIV-1_{NL4-3} (compare Fig. 1 and 4). A similar difference was observed when comparing MA from R7 and R9 virions (compare Fig. 1 and 5). This most likely reflects small differences in the NL4-3 and HXB2 MA sequences, which probably affect the sensitivity of the proteins to digestion by trypsin. However, the Nef dependence of MA serine phosphorylation was observed for both R7 and R9 virions (data not shown).

The human serine kinase PAK65 can phosphorylate MA in vitro. Recent results indicate that the Nef-associated serine/threonine kinase is related to the p21-activated kinase (PAK) family (29, 36, 42). Based on this premise, the ability of recombinant human PAK65 (32) to phosphorylate MA in an in vitro kinase assay was evaluated (Fig. 5). When PAK was activated by incubation with the small GTP-binding protein cdc42, it autophosphorylated and could use MBP as a substrate (Fig. 5A, lane 2). Activated PAK also efficiently mediated the serine phosphorylation of recombinant MA (Fig. 5A, lane 4), yielding a tryptic phosphopeptide pattern reminiscent of that observed with virion-associated MA (Fig. 5B), even though additional phosphopeptides were observed, perhaps reflecting the extreme efficiency and thereby partial lack of specificity of the in vitro kinase assay. Several other recombinant protein kinases were tested for their ability to phosphorylate MA in vitro.

FIG. 4. In vitro serine phosphorylation of HIV-1 MA by a Nef-associated kinase. (A) Lysates from CEM cells constitutively expressing nonmyristoylated (lane 1) or wild-type (lanes 2 and 3) forms of Nef or from control CEM cells (lane 4) were immunoprecipitated with Nef-specific antiserum (3). Immune complexes were subjected to an in vitro kinase assay with [γ - 32 P]ATP without (top) or with (middle) addition of recombinant MA. After SDS-PAGE and autoradiography, a 62-kDa phosphoprotein (top, arrow) and MA kinase activity (middle) were detected in precipitates from wild-type Nef-expressing cells (lanes 2 and 3). A Western blot analysis (bottom) controlled for Nef production. (B) Two-dimensional phosphoamino acid analysis of 32 P-labelled MA from panel A demonstrates that only serine is phosphorylated. (C) Two-dimensional tryptic phosphopeptide analysis of 32 P-labelled MA from panel A reveals a pattern comparable to that observed in virions. Unlabelled arrows indicate phosphopeptides.

Mitogen-activated protein kinase was inactive, whereas protein kinase A (PKA) and casein kinase II did phosphorylate MA but induced phosphopeptide patterns that markedly differed from that observed with MA molecules purified from virions (data not shown). PKC, which has been suggested to mediate MA phosphorylation (8, 52), could also use the viral protein as a substrate. However, PKC targeted phosphoacceptor sites completely distinct from the ones modified in virion MA (Fig. 5C). These data, while not formally identifying the MA serine kinase, lend further support to a model in which the Nef-associated kinase is the mediator of MA serine phosphorylation and is related to hPAK65.

DISCUSSION

This study reveals that an accessory protein of HIV-1, Nef, is involved in stimulating the serine phosphorylation of one of the virus structural components, MA. Whereas the levels of phosphoserine-containing MA were significantly decreased in *nef*-defective virions, Nef did not stimulate MA tyrosine phosphorylation nor the additional serine phosphorylation of MA that takes place after viral entry.

Although the mechanism by which Nef enhances MA serine phosphorylation is yet undefined, several lines of evidence incriminated the Nef-associated cellular serine kinase. First, mutations in Nef which prevent the recruitment of this kinase,

FIG. 5. In vitro serine phosphorylation of HIV-1 MA by hPAK65 and PKC. (A) Top: In vitro phosphorylation of recombinant MA by cdc42-activated (lane 4) but not by unactivated (lane 3) human PAK65. Reactions were done in the presence of [γ - 32 P]ATP and analyzed by SDS-PAGE and autoradiography. MBP was used as a positive control substrate for activated hPAK65 (lanes 1 and 2). Bottom: PKC-mediated in vitro phosphorylation assay, similarly analyzed, using histone H1 (lane 2) or recombinant HIV-1 MA (lane 3) as substrates; lane 1 contained no substrate. (B) Two-dimensional tryptic phosphopeptide analysis of 32 P-labelled MA purified from R7 virions (top) or phosphorylated in vitro with hPAK65 (middle) or PKC (bottom). Unlabelled arrows indicate phosphopeptides.

such as ones targeting the myristoylation signal or a critical arginine in Nef, also impaired MA serine phosphorylation. Second, a cellular protein kinase activity that coimmunoprecipitates with Nef could phosphorylate MA on serine in vitro, by using the same major phosphoacceptors as observed in vivo. Finally, recombinant hPAK65, recently proposed to be related to the Nef-associated kinase, could use MA as an in vitro substrate, producing a phosphopeptide pattern reminiscent of, although not identical to, that observed in MA molecules purified from HIV-1 particles. In contrast, several other recombinant protein kinases failed to exhibit such an ability, including mitogen-activated protein kinase, casein kinase II, PKA, and PKC. Of note, it was previously demonstrated that PKC can phosphorylate MA on serine in vitro and in transfected COS cells (8, 52). Although our analyses confirmed that PKC can use MA as a substrate, they also revealed that the resulting phosphopeptide pattern differs markedly from that observed with virion MA.

Because MA kinase activity can be detected in virions (6, 18), we asked whether the particle incorporation of this enzyme was Nef dependent. When highly purified wild-type, Δ Nef, or Nef_{RR106AA} viruses were used as sources of kinase activity, recombinant MA was efficiently phosphorylated on serine and tyrosine, with no discernable difference between the viruses (data not shown). Also, similar levels of phosphorylation were noted on endogenous viral MA molecules immunoprecipitated after performing an in vitro kinase reaction on purified virions (data not shown). Although it is possible that

the virion-associated serine kinase activity detected in these assays does not correspond to the enzyme responsible for modifying MA *in vivo*, these results suggest that Nef does not act by recruiting the MA serine kinase in virions. Instead, Nef could activate this enzyme in the context of assembling particles, promote its interaction with MA, or even stimulate the preferential incorporation of serine-phosphorylated MA molecules into virions.

While MA tyrosine phosphorylation has been shown to facilitate HIV-1 nuclear import (6, 18, 19), the functional significance of MA serine phosphorylation remains unclear. The treatment of virus producer cells with the serine/threonine kinase inhibitor H7 was recently shown to decrease markedly the infectivity of HIV-1 in dividing as well as nondividing cells (6). This effect correlated with reduced levels of MA translocation into the nucleus of target cells. Because a virus expressing a nonmyristoylated form of MA was unaffected by the drug, it was proposed that serine phosphorylation triggers the membrane release of MA, hence allowing the viral nucleoprotein complex to migrate first to the cytosol and then to the nucleus. Nevertheless, these results must be interpreted with caution, because inhibitors of serine/threonine kinases are likely to interfere with the phosphorylation of CA, another major phosphoprotein of HIV-1 virions (12) (Fig. 2) which plays an important role during the early steps of infection (50).

It is tempting to propose that the serine phosphorylation of MA might contribute to the positive effect of Nef on HIV-1 replication. However, HIV-1 mutants expressing the Nef_{RR106AA} variant were found to be only moderately impaired in single-round infectivity assays, when produced either from 293 or from CEM cells, as they exhibited infectious titers that were approximately 30% those of wild type, that is, from 3- to 30-fold higher than those of *nef*-deleted viruses (data not shown). Of note, MA phosphoserine-defective variants could exhibit a phenotype distinct from that of Nef-mutated virions, because some degree of MA serine phosphorylation is conserved in the absence of Nef. Our analyses have allowed us so far to detect at least five phosphoserine residues in HIV-1 MA, although their exact mapping is still in progress. Ultimately, the identification of these phosphoacceptors will be required to elucidate fully the role of MA serine phosphorylation, as well as to explore further the connection between this process and the observed effect of Nef on HIV-1 replication.

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