JOURNAL OF VIROLOGY, Sept. 1997, p. 6834–6841 Vol. 71, No. 9
0022-538X/97/$04.00+0

Human Immunodeficiency Virus Matrix Tyrosine Phosphorylation: Characterization of the Kinase and Its Substrate Requirements

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Received 22 January 1997/Accepted 13 June 1997

During virus assembly, a subset of human immunodeficiency virus (HIV) matrix (MA) molecules is phosphorylated on C-terminal tyrosine. This modification facilitates infection of nondividing cells by allowing for the recruitment of the karyophilic MA into the viral core and preintegration complex. MA tyrosine phosphorylation is accomplished by a cellular protein kinase which is incorporated into virions. In this study, we have investigated the nature of this enzyme as well as the determinants of MA necessary for its phosphorylation. Employing an in vitro kinase assay, we found that the MA tyrosine kinase activity is present in various cultured cell lines including CEM and SupT1 T-lymphoid cells, Namalwa B cells, 293 and CV-1 kidney fibroblasts, and P4 HeLa cells. In addition, it could be detected in platelets, macrophages, and activated peripheral blood lymphocytes (PBLs) but not in erythrocytes and resting PBLs isolated from human blood. Subcellular localization of the kinase activity by cell fractionation demonstrated that it is enriched in cellular membranes. In HIV type 2 (HIV-2) particles, the MA tyrosine kinase is associated with the inner leaflet of the viral membrane, while the tyrosine-phosphorylated MA is localized to the core. Individual mutations of each of the last eight residues immediately upstream of the C-terminal tyrosine (Y132) of HIV-1 MA did not prevent Y132 phosphorylation, suggesting that the kinase does not require a highly specific sequence adjacent to the C-terminal tyrosine. Confirming this, we found that the MA of murine leukemia virus, the sequence of which is only moderately homologous to that of HIV-1 and HIV-2 MA, is also C-terminally tyrosine phosphorylated.

The matrix (MA) protein of human immunodeficiency virus type 1 (HIV-1) is the N-terminal cleavage product of the $Pr55^{Gag}$ polyprotein. MA has been demonstrated to perform several functions at both early and late stages of the retroviral life cycle. In the producer cell, MA exists in the context of the Gag precursor, which is translated from unspliced genomic RNA on membrane-free polyribosomes (48). Gag precursors assemble at the cytoplasmic face of the plasma membrane (21) and have been demonstrated to be sufficient for virus assembly since, in the absence of any other viral components, virus-like particles are formed (23, 26, 39, 40).

Several lines of evidence have implicated domains of MA in proper viral assembly. Like most retroviral MA proteins, HIV MA is cotranslationally modified by N-terminal myristoylation (37). The myristate group provides a hydrophobic interaction with the cell plasma membrane, thereby allowing the membrane attachment of $Pr55^{Gag}$. Mutations that block this modification completely inhibit virus assembly at the membrane (37, 47). But while myristoylation is necessary for intracellular transport and membrane attachment of Gag, it is not sufficient. The polybasic domain of MA (amino acids 15 to 30) appears to be important for both transport and stable membrane association of $Pr55^{Gag}$, presumably through electrostatic interaction with negatively charged membrane phospholipids (42, 52, 53). In addition, large deletions in MA within the context of Gag have resulted in the intracellular budding of virus-like particles (12, 29); specifically, disruption of the MA domain from amino acids 84 to 88 appears to be implicated in this redirection of virus budding (16).

Envelope incorporation is another role of MA during virus assembly and one which is very sensitive to minor alterations in MA. Small missense or deletion mutations in the MA region encoding the first 100 residues result in the production of virions that lack both surface glycoprotein gp120 and transmembrane glycoprotein gp41 (11, 51). Specifically, amino acids 12, 30, and 34 of HIV-1 MA appear to be important for interaction with gp41 (15). An analysis of chimeric virus-like particles which contained only the HIV-1 MA and the capsid (CA) and nucleocapsid (NC) proteins of visna virus, but still incorporated HIV-1 envelope, demonstrated that MA is both necessary and sufficient for selective envelope incorporation (11). Confirming this, a recent in vitro study demonstrated a direct interaction between HIV-1 MA and the cytoplasmic domain of HIV-1 envelope but not the envelope domains of other retroviruses (9).

Concomitant with or soon after budding of the immature HIV-1 virions from producer cells, $Pr55^{Gag}$ is cleaved by the viral protease into the MA, CA, NC, and p6 proteins. In mature virions, the majority of MA is localized to the inside of the viral lipid bilayer, corresponding to what was once the cell plasma membrane, arranged in a continuous shell approximately 7 nm thick (21, 22). The typical lentivirus cone-shaped mature core, enclosed by the CA, is located inside the MA layer and is separated from it by a space of about 5 to 6 nm (21). The viral genomic RNA resides within the capsid shell complexed with NC and the viral enzymes reverse transcriptase (RT) and integrase (IN). Studies of HIV-2 and the related simian immunodeficiency virus particles have demonstrated that a fraction of MA is associated with these mature viral cores (20, 49). Because of its subsequent role in nuclear import, Vpr is likely to be associated with HIV-1 cores as well.

MA also performs several functions after the virus enters target cells. The C terminus of MA has been proposed to play a role in viral entry into target cells (50). During entry, the viral envelope and the cell plasma membrane fuse, resulting in the

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release of the viral core into the cell cytoplasm. The MA that was associated with the viral envelope remains membrane associated (20). The core subsequently undergoes a little-understood process to yield an uncoated nucleoprotein complex. This complex contains the viral genome as well as IN, RT, NC, Vpr, and the fraction of MA that was associated with the viral core (6, 13, 20, 32). The importance of MA in nucleoprotein complexes is best revealed during infection of nondividing cells and in the absence of a functional Vpr protein (4, 45). In this context, the nuclear localization signal of MA, a charged region which spans amino acids 25 to 33, directs the transport of the HIV-1 nucleoprotein complex into the nucleus (4). This transport is a rapid ATP-dependent process (5) and occurs through the specific recognition of the MA nuclear localization signal by members of the karyopherin-α family (18).

Studies performed in our laboratory have shown that the transition of MA function from virion assembly in producer cells to nuclear import in target cells is accomplished through phosphorylation. Specifically, a subset of MA molecules is phosphorylated on C-terminal tyrosine at the stage of virion formation (19). This phosphorylation occurs after $Pr55^{Gag}$ cleavage and is accomplished by a cellular protein kinase. In mature HIV-2 virions, tyrosine-phosphorylated MA molecules are localized exclusively to the viral cores. This localization is due to a phosphotyrosine (pTyr)-dependent association of MA with IN (20). After target cell infection, MA and IN remain associated and can be coimmunoprecipitated from the viral nucleoprotein complex. MA molecules in which the C-terminal tyrosine has been changed to a phenylalanine are not found in viral cores and remain associated with the infected cell membrane (20). Furthermore, at low multiplicities of infection and in the absence of Vpr, HIV-1 virions containing this non-tyrosine-phosphorylated MA mutant (Y132F) cannot infect terminally differentiated macrophages as efficiently as those with wild-type (WT) MA (16, 17). Corroborating this data, Bukrinskaya et al. demonstrated that HIV-1 virions produced in the presence of the tyrosine kinase inhibitors genistein and herbimycin are impaired for infection of G_2-arrested target cells (3). In addition, they found that the non-tyrosine-phosphorylated MA is defective for nuclear localization in target cells and instead remains associated with the membranes and cytosol.

In contrast, Freed et al. failed to detect a replicative defect of the MA_{Y132F} mutant in macrophage growth curves (14). One possible explanation for this discrepancy between their data and ours could be a difference in multiplicity of infection. In agreement with their data, we indeed observed that, at a high input of virus, the defective phenotype of viruses mutated either in the C-terminal tyrosine or in the nuclear localization signal of MA can be masked (17, 44). This finding in conjunction with recent data from our laboratory (17) has implicated yet a third player in viral nuclear import, the HIV-1 IN protein. Thus, it is possible that in high-multiplicity-of-infection inocula there is enough virus capable of infecting macrophages through an IN-dependent and MA-Vpr-independent pathway to allow productive viral replication.

The current study was undertaken to characterize the tyrosine kinase responsible for MA phosphorylation as well as the requirements at the level of its substrate. The results of our investigation are presented here.

MATERIALS AND METHODS

DNA constructions. The $HIV\text{-}2_{ROD10}$ construct was a gift from M. Emerman and expresses the $HIV\text{-}2_{ROD}$ proviral DNA. The R7 plasmid, which encodes HIV-1, has been described elsewhere (27). $R7MA_{S125A}$, $R7MA_{S129A}$, and $R7MA_{S125A/S129A}$ were generated by PCR-mediated mutagenesis of the gene encoding MA in the context of the R7 construct. Plasmid R7MASTOP was created by introducing a stop codon immediately following the MA-encoding gene reading frame in R7. R7MASTOP therefore encodes full-length MA, envelope, Tat, Rev, Vif, and Nef but not other Gag or Pol proteins. The $R7MA_{H124A}$STOP, $R7MA_{N126A}$STOP, $R7MA_{Q127A}$STOP, $R7MA_{V128A}$STOP, $R7MA_{Q130A}$STOP, $R7MA_{N131A}$STOP, and $R7MA_{Y132F}$STOP constructs were generated by PCR-mediated mutagenesis of the gene encoding MA in the context of R7MASTOP. The GST-MA, GST-MA_{Y132F}, GST-$MA_{119–132}$, GST-VSQNY, and GST-Y expression vectors were created by PCR cloning of sequences encoding full-length HIV-1 MA and MA_{Y132F} as well as the MA C-terminal 14, 5, and final amino acids into pGEX-2T (Pharmacia) to produce glutathione *S*-transferase (GST) fusion proteins. The pCMV-GAGPOL and pMD.G plasmids have been previously described (34). The former encodes the Gag and Pol proteins of murine leukemia virus (MLV), and the latter encodes the G glycoprotein of vesicular stomatitis virus (VSV). Plasmid pCLMFG-LacZ, previously described, is an MLV-based retroviral vector that encodes β-galactosidase (35). The pCMV-GAG(MA_{Y131F})POL plasmid was generated by PCR mutagenesis of the gene encoding MA in the context of the pCMV-GAGPOL parent vector. WT and tyrosine phosphorylation mutant (Y132F) recombinant HIV-1 MA with N-terminal histidine tags were produced in *Escherichia coli* with the bacterial expression vector pET-15b (Novagen) and purified by affinity chromatography on nickel-Sepharose columns according to the instructions of the manufacturer.

Cells. The CEM and SupT1 human T-cell lines as well as the Epstein-Barr virus-transformed Namalwa human B-cell line were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. HeLa-derived P4 cells (8) were a gift from F. Clavel. 293T cells are a derivative of the 293 human kidney cell line that stably expresses the simian virus 40 large T antigen. The P4, 293, 293T, and CV-1 monkey kidney cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The NIH 3T3-MLV and NIH 3T3-MLV(MA_{HIV}) cell lines (10) were a gift from M. Emerman and constitutively produce MLV with either WT MLV MA or HIV-1 MA with the last four amino acids of MLV MA, respectively. The NIH 3T3 lines and rat 208F cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Blood cell populations including erythrocytes, platelets, and peripheral blood mononuclear cells were isolated by banding of whole blood obtained from seronegative donors on Ficoll-Paque (Pharmacia LKB). Erythrocytes were further purified by two rounds of gravity filtration through a 2% gelatin solution (in phosphate-buffered saline [PBS]) for 30 min at 37°C. Contaminating cells were removed from the platelet population by repeated low-speed centrifugation ($200 \times g$) in a Sorvall tabletop centrifuge. The platelets, which remained in suspension during the low-speed centrifugation, were subsequently pelleted by centrifugation at $1,800 \times g$ in the Sorvall centrifuge. Peripheral blood mononuclear cells were cultured in RPMI 1640 medium with 10% fetal calf serum, and the monocyte-derived macrophages were removed by overnight adherence to plastic. They were subsequently cultured for 2 weeks. The resulting peripheral blood lymphocytes (PBLs) were removed and split into two flasks. One flask was left untreated; these unstimulated PBLs were cultured for a week. The PBLs in the other flask were activated by treatment with 3 μg of phytohemagglutinin per ml for 48 h and maintained for a week in medium supplemented with 10 U of recombinant human interleukin-2 (Bethesda Research Laboratories) per ml.

Transfections, virus preparation, and infections. 293T cells were transfected by the calcium phosphate method as previously described (2). Two days following transfection, the 293T cells were lysed and supernatants were harvested for virus preparation. For cell lysate preparation, 8×10^6 transfected cells were washed twice with PBS and then resuspended in 500 μl of cell lysis buffer (10 mM NaCl, 10 mM Tris-HCl [pH 7.5], 0.5% Nonidet P-40 [NP-40], 1 mM sodium orthovanadate, 100 μg of phenylmethylsulfonyl fluoride per ml, 1 μg of aprotinin per ml, 1 μg of pepstatin A per ml, 2 μg of leupeptin per ml) for 5 min on ice. The lysates were subsequently ultracentrifuged at $14,000 \times g$ in a tabletop microcentrifuge for 5 min at 4°C to remove nuclei, and the supernatants were harvested and stored at −75°C until needed. Total protein content of the lysates was measured with the bicinchoninic acid assay (Pierce). HIV-1 virions and VSV-G pseudotyped MLV retroviral particles from transfected 293T cells and MLV virions from NIH 3T3 cells were prepared by filtration of the virus-containing supernatant through a 0.45-μm-pore-size nitrocellulose membrane followed by ultracentrifugation through a 20% (wt/vol) sucrose cushion at 26,000 rpm in an SW28 rotor (Beckman) for 1.5 h at 4°C. Pellets were resuspended in 250 μl of PBS for 2 h at 4°C and stored at −75°C. HIV-1-containing samples were analyzed for p24 (CA) content by enzyme-linked immunoabsorbent assay (DuPont). Virion content of the MLV-containing samples was quantitated by a modified exogenous reverse transcription assay originally described by Goff et al. (24). Briefly, 10 μl of concentrated virus was added to 20 μl of assay buffer containing 50 mM Tris-HCl (pH 7.9), 75 mM KCl, 2 mM dithiothreitol, 5 mM $MnCl_2$, 25 μg of poly(A) · $oligo(dT)_{12–18}$ (Pharmacia), 0.05% NP-40, and 50 μCi of [^{3}H]TTP per ml and incubated for 2 h at 37°C. Then the reaction mixtures were spotted onto 2.3-cm-diameter DE81 paper circles (Whatman), and the disks were washed three times for 5 min in 2× SSC (300 mM NaCl, 30 mM sodium citrate) and two times for 5 min in 95% ethanol. Once the filters were air dried, tritium incorporation was determined by liquid scintillation counting. To determine the infectivity of the VSV-G pseudotyped MLV retroviral particles containing a β-galactosidase transfer vector, 4×10^4 rat 208F cells were plated in 12-mm wells

and infected 24 h later with 500 μl of virus-containing supernatant (diluted to 50 to 240 cpm of RT activity per ml) supplemented with Polybrene (8 μg/ml). Twelve hours after infection, 2 ml of complete medium was added, and 2 days postinfection, cells were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The number of blue foci was subsequently counted under the light microscope.

Preparation of extracts used in the in vitro kinase assay. All cytoplasmic extracts used in the kinase assay were prepared by washing 10^7 cells extensively with PBS and resuspending them in 300 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 20 mM $MnCl_2$, 50 mM NaCl, 1 mM sodium orthovanadate, 0.1% NP-40, 10 mM $MgCl_2$) for 10 min on ice. Nuclei were removed by centrifugation at 14,000 × *g* in a tabletop microcentrifuge for 5 min at 4°C, and the supernatants were harvested and stored at −75°C. Membrane, cytosol, and nuclear fractions were prepared from CEM cells according to the method of Lin et al. (31). Briefly, 5×10^8 cells were resuspended in 5 ml of homogenization buffer (50 mM mannitol, 10 mM HEPES [pH 7.4], 1 mM sodium orthovanadate) and incubated for 10 min on ice. Samples were then aspirated through a blunt 23-gauge needle six times. $CaCl_2$ was subsequently added to the samples to a final concentration of 10 mM, and nuclei were removed by centrifugation at 14,000 × *g* in a tabletop microcentrifuge for 5 min. The nuclear pellet was solubilized in 500 μl of high-salt buffer (20 mM potassium-HEPES [pH 7.8], 500 μM $MgCl_2$, 500 mM potassium acetate, 500 μM dithiothreitol, 1 mM sodium orthovanadate), and the membranes were separated from the cytosol by ultracentrifugation at 32,000 rpm in an SW55 rotor (Beckman) for 30 min at 4°C. The resulting membrane pellet was resuspended in 100 μl of NTE buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM sodium orthovanadate). The nuclear, membrane, and cytosolic extracts were stored at −75°C. HIV-2_{ROD} viral membrane and core fractions were prepared as previously described (20). Briefly, concentrated HIV-2_{ROD} virions produced from acutely infected CEM cells were resuspended in 500 μl of PBS containing 1% Igepal CO-630 (Rhone-Poulenc) and immediately overlaid onto a 20 to 60% sucrose gradient. After centrifugation at 20,000 rpm in an SW40 rotor (Beckman) for 24 h at 4°C, fractions were collected and tested for the content of various viral proteins. Specifically, Western blot analysis using monoclonal antibodies against HIV-2 envelope (Intracel) and CA (Advanced Biotechnologies, Inc.) was performed to assess which fractions contained HIV-2 cores and which contained viral membranes. Where indicated, HIV-2 virions were first treated with 1 mg of proteinase K (Sigma) per ml for 15 min at room temperature before concentration and subsequent detergent treatment.

In vitro kinase assay. Extracts to be tested (50 to 150 μg) in up to 100 μl of assay buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM sodium orthovanadate, 0.1% NP-40, 5 mM $MnCl_2$) were mixed with 3 to 5 μg of recombinant HIV-1 MA (MA_{WT} or tyrosine mutant MA_{Y132F}) in the presence of 5 mM ATP and incubated at 30°C for 1 h. Recombinant MA was then purified by affinity chromatography on nickel-Sepharose beads according to the manufacturer's instructions (Novagen) and subjected to Western blot analysis. Recombinant mouse Src expressed in Sf9 insect cells was a gift from M. Broome and T. Hunter, The Salk Institute for Biological Studies, La Jolla, Calif.

Expression of GST fusion proteins in TKX1 bacteria. GST-MA, GST-MA_{Y132F}, GST-$MA_{119–132}$, GST-VSQNY, GST-Y, and GST proteins were expressed by transformation of TKX1 bacteria (Stratagene) with the appropriate pGEX-2T vectors. Protein expression followed by tyrosine kinase induction was according to the manufacturer's instructions. The fusion proteins were isolated on GST affinity columns, eluted with 10 mM glutathione, and stored at −75°C.

Western blot (immunoblot) detection. Recombinant MA subjected to the in vitro kinase assay, cell extracts, virions, and GST-MA fusion proteins were assayed for pTyr-containing MA. Proteins were separated on sodium dodecyl sulfate–15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Micron Separations Inc.) in a buffer containing 25 mM Tris-HCl (pH 8.0), 192 mM glycine, 0.035% sodium dodecyl sulfate, and 20% methanol. Membranes were incubated with a 1:1,000 dilution of rabbit anti-pTyr (a gift from B. Sefton, Salk Institute), a 1:1,000 dilution of monoclonal anti-HIV-1 MA (Advanced Biotechnologies, Inc.), a 1:200 dilution of monoclonal anti-HIV-1 CA (Genzyme), a 1:1,000 dilution of monoclonal anti-GST (Santa Cruz Biotechnology, Inc.), or a 1:500 dilution of goat anti-Rauscher leukemia virus (RLV) p15 antiserum (obtained through the National Cancer Institute from Quality Biotech Inc.). The membranes were washed in a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20. Detection was performed with a 1:2,000 dilution of horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat (Dako) immunoglobulin by enhanced chemiluminescence (ECL Western blotting kit; Amersham) according to the manufacturer's instructions.

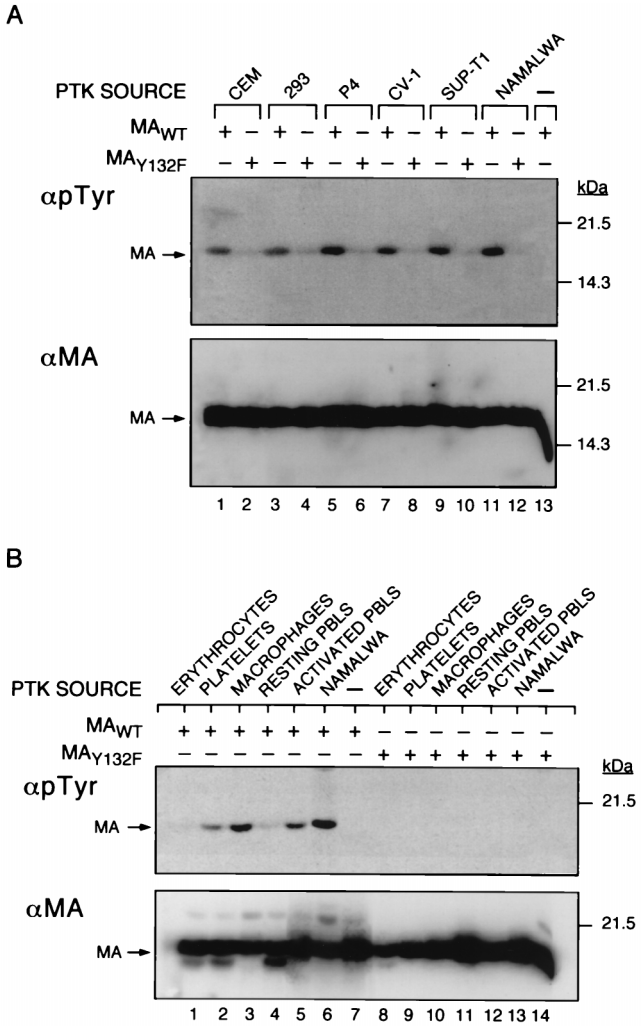

FIG. 1. MA tyrosine kinase activity is present in various cell lines and platelets, macrophages, and activated PBLs. (A) In vitro kinase assay using recombinant MA_{WT} or MA_{Y132F} (5 μg) as substrates and cell line extracts (100 μg of protein) as protein tyrosine kinase (PTK) sources. Following the kinase reactions, MA was isolated on 50 μl of nickel-charged resin and then analyzed by Western blotting to monitor tyrosine phosphorylation. Locations of molecular mass markers are shown on the right. (Upper panel) Recovered MA_{WT} and MA_{Y132F} from the kinase reactions probed with a pTyr-specific antiserum. Lane 13, negative control in which MA_{WT} was not incubated with a tyrosine kinase source. (Lower panel) The same blot probed with an anti-MA antibody to verify that equal amounts of MA_{WT} and MA_{Y132F} were recovered from each reaction. (B) In vitro kinase assay testing extracts (150 μg of protein) prepared from human blood cell populations for their ability to phosphorylate MA_{WT} and MA_{Y132F} (5 μg). A molecular mass marker is shown on the right. (Upper panel) Anti-pTyr probing of the recovered MA proteins following incubation with the extracts. Lane 6, positive control, MA_{WT} with Namalwa cytoplasmic extract; lane 7, negative control, MA_{WT} with no kinase source. (Lower panel) Subsequent probing of the blot with an anti-MA antibody.

RESULTS

Detection of MA tyrosine kinase activity in cell lines and primary blood cells. To assess the distribution of the MA tyrosine kinase, various cultured cell lines were tested in the in vitro kinase assay. Cytoplasmic extracts were prepared from human cell lines of different tissue origins including CEM and SupT1 T-lymphoid cells, Namalwa B cells, 293 kidney cells, and P4 cervical carcinoma cells. In addition, a line from a different species, CV-1 monkey kidney, was tested. Extracts were normalized for total protein content and then incubated with recombinant histidine-tagged MA_{WT} or the control MA_{Y132F} in the presence of ATP to allow tyrosine phosphorylation. MA was then isolated by virtue of its tag, and its phosphorylation was monitored by anti-pTyr immunoblotting. All of the lines tested possessed tyrosine kinase activity capable of phosphorylating MA_{WT} in the in vitro reaction (Fig. 1A, upper panel, lanes 1, 3, 5, 7, 9, and 11). This phosphorylation occurred specifically on Y132, the C-terminal residue of MA,

as evidenced by the fact that incubation of the same extracts with recombinant MA_{Y132F} resulted in no detectable tyrosine phosphorylation (upper panel, lanes 2, 4, 6, 8, 10, and 12). In the absence of a kinase source, MA_{WT} did not react with the anti-pTyr antibody (upper panel, lane 13), revealing the specificity of the antibody for pTyr and not for tyrosine. To ensure that equal amounts of MA substrate were used and subsequently recovered in all of the reactions, the blot was then probed with an anti-MA antibody (bottom panel). Because the Namalwa cell extract repeatedly gave stronger MA pTyr signals than the other extracts, it was used as a positive control in subsequent experiments.

Next, cell populations isolated from human blood were tested in the in vitro kinase assay. We were particularly interested in determining whether the natural targets of HIV infection, macrophages and activated PBLs, possess MA tyrosine kinase activity. Erythrocytes, platelets, macrophages, activated PBLs, and resting PBLs were prepared from buffy coats. Extracts of the cell populations were normalized for total protein content and then used as potential kinase donors in the in vitro assay. Immunoblot analysis indicated that MA_{WT} was tyrosine phosphorylated when incubated with platelet, macrophage, and activated PBL extracts (Fig. 1B, upper panel, lanes 2, 3, and 5) but not with erythrocyte or resting PBL extracts (upper panel, lanes 1 and 4). The control, MA_{Y132F}, was not tyrosine phosphorylated by any of the blood cell populations (upper panel, lanes 8 through 12). Thus, it appeared that the targets of HIV infection in vivo, macrophages and activated PBLs, as well as platelets and various cell lines all contain a tyrosine kinase capable of phosphorylating MA at Y132.

Subcellular and subviral localization of the MA tyrosine kinase. We next examined the subcellular localization of the MA kinase in CEM cells. To do so, cells were separated into membrane, cytosol, and nuclear fractions. The fractions were then normalized for total protein content and tested in the in vitro kinase assay with MA_{WT} as the substrate. Cellular membranes contained readily detectable tyrosine kinase activity while the cytosol and nuclear fractions did not (Fig. 2, upper panel). This finding was compatible with our earlier finding that tyrosine phosphorylation of MA in cells requires its membrane association (19).

To investigate the subviral localization of the kinase, HIV-2 particles were fractionated into viral cores and viral membranes by partial lysis of the virions followed by centrifugation through a sucrose density gradient. HIV-2 was used for this experiment because HIV-1 particles are unstable in the presence of detergent and do not yield large quantities of cores as do HIV-2 particles. As previously described (20), HIV-2 viral cores exhibit a density of 1.24 to 1.25 g/cm^3 and contain CA, IN, RT and tyrosine-phosphorylated MA but no envelope. In contrast, viral membranes are found at the top of the gradient with envelope. Fractions (approximately 500 μl) containing cores and membranes were harvested from the gradient and dialyzed against PBS for 12 h at 4°C. Then, 50 μl of each was tested in the in vitro kinase assay for MA tyrosine kinase activity. Intact HIV-2 virions contained kinase activity (Fig. 3A, lane 5) as previously reported (19), and fractionation of these virions showed that the kinase activity is localized to the membranes rather than the cores (lanes 6 and 7). Next, virions were treated with proteinase K prior to subviral fractionation to cleave off any protein domains on the outside of the viral membranes. These viral membranes were then tested in the in vitro kinase assay. Proteinase treatment did not abolish the tyrosine kinase activity (Fig. 3B, lane 5), consistent with the kinase being localized to the inside of the viral lipid bilayer. The efficacy of proteinase K cleavage was monitored by anti-

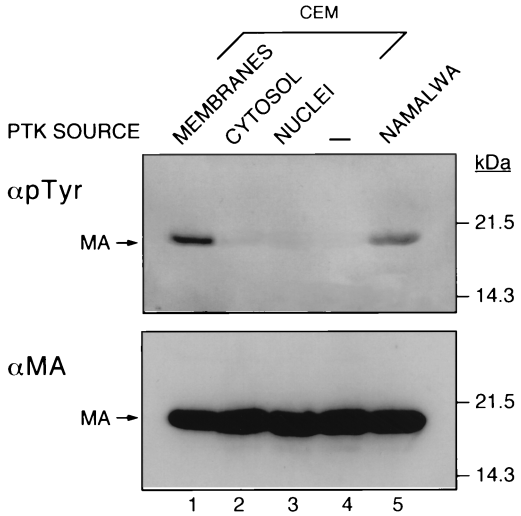

FIG. 2. Cellular membranes are enriched for MA tyrosine kinase activity. CEM T cells were fractionated into membrane, cytosol, and nuclear compartments, and the fractions (50 μg of protein) were incubated with MA_{WT} (3 μg) in kinase assay buffer. Immunoblot analysis was then performed on MA_{WT}. Molecular mass markers are shown on the right. (Upper panel) Reaction mixtures were probed with anti-pTyr antiserum. Lane 4, negative control with no kinase source; lane 5, positive control with Namalwa cytoplasmic extract. (Lower panel) Equivalent amounts of MA_{WT} were recovered from each reaction as assessed by anti-MA immunodetection. PTK, protein tyrosine kinase.

envelope immunodepletion of viral membranes. Membranes from virions that were first treated with proteinase K could not be depleted with an antienvelope antibody (lane 7), indicating that gp105 had been cleaved. In contrast, membranes from untreated virions that retained gp105 could be immunodepleted (lane 6).

Determinants of MA Y132 phosphorylation. Phosphorylation of a C-terminal tyrosine is an uncommon event. We were thus interested in investigating the determinants of HIV-1 MA necessary for Y132 phosphorylation. Specifically, we asked whether amino acids in the C terminus immediately upstream of Y132 were critical for its phosphorylation. To address this question, we constructed vectors encoding MA mutants in which individual residues were changed to alanine (H124A, N126A, Q127A, V128A, Q130A, and N131A). These MA mutants were expressed in 293T cells, and their Y132 phosphorylation states were monitored by Western blot analysis with the anti-pTyr antibody. In this context, all of the mutants were expressed except for Q127A (Fig. 4A, lower panel) and were also tyrosine phosphorylated (upper panel). Next, serines 125 and 129 of HIV-1 MA were mutated to alanine individually or together in the context of the proviral R7 construct. The R7 (WT) plasmid and the mutant constructs (S125A, S129A, and S125A/S129A) were then transfected into 293T cells. The resulting virions were concentrated from supernatants and analyzed by Western blotting for tyrosine-phosphorylated MA. Neither of the single mutations, S125A and S129A, nor the double mutation, S125A/S129A, abolished Y132 phosphorylation (Fig. 4B, upper panel). Therefore, residues adjacent to Y132F did not appear to be essential for kinase recognition or phosphorylation.

Examination of the minimal domain necessary for Y132 phosphorylation in vitro. We next sought to determine the minimal domain of the HIV-1 MA C terminus necessary for Y132 phosphorylation. To do so, we created vectors encoding the GST protein fused to the last 14 amino acids of MA

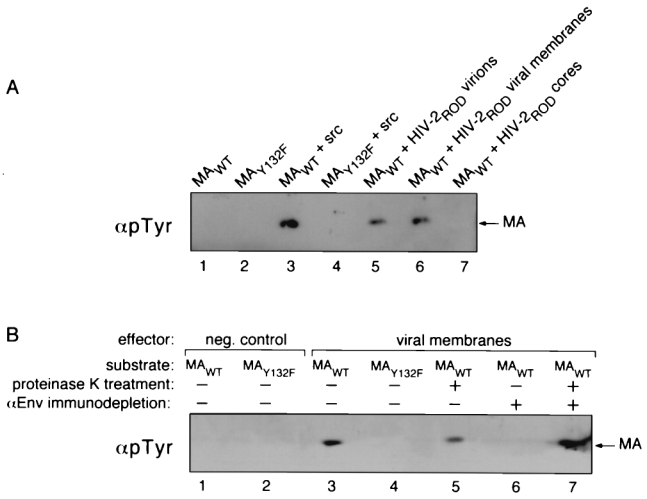

FIG. 3. MA tyrosine kinase activity is localized to HIV-2 viral membranes. HIV-2 virions were fractionated into viral membranes and cores, and the fractions were tested in the in vitro kinase assay with MA_{WT} and MA_{Y132F} (5 μg). (A) Western blot probing with anti-pTyr antiserum following the kinase reactions. Lanes 1 and 2, negative controls, MA_{WT} and MA_{Y132F}, respectively, treated with no kinase source; lanes 3 and 4, MA_{WT} and MA_{Y132F}, respectively, treated with recombinant Src (20 ng); lanes 5 through 7, MA_{WT} incubated with HIV-2 virion, membrane, and core extracts, respectively. (B) HIV-2 particles were first treated with proteinase K before fractionation to digest extravirion proteins. Viral membranes were then prepared from the treated virions, and the efficacy of proteinase K treatment was monitored by subsequent anti-envelope immunodepletion. These viral membrane samples were then used in the in vitro assay with MA_{WT} and MA_{Y132F} (5 μg). Tyrosine phosphorylation of the MA substrates was assayed by anti-pTyr immunodetection. Lanes 1 and 2, negative controls in which MA_{WT} and MA_{Y132F}, respectively, were treated with no kinase source; lanes 3 and 4, MA_{WT} and MA_{Y132F}, respectively, incubated with viral membranes; lane 5, MA_{WT} incubated with proteinase K-treated viral membranes; lane 6, MA_{WT} incubated with viral membranes not proteinase K treated before anti-envelope immunodepletion; lane 7, MA_{WT} incubated with viral membranes first proteinase K treated and then subjected to anti-envelope immunodepletion.

($GST\text{-}MA_{119–132}$), the last 5 residues (GST-VSQNY), or just the terminal tyrosine (GST-Y). These constructs along with control vectors encoding GST or GST fused to full-length MA (GST-MA and $GST\text{-}MA_{Y132F}$) were transformed into TKX1 bacteria. The TKX1 bacteria encode the catalytic domain of Elk, a brain-specific receptor tyrosine kinase. Expression of the GST fusion proteins was followed by induction of the Elk kinase, and the fusion proteins were subsequently isolated and subjected to Western blot analysis (Fig. 5). GST-MA but not the control $GST\text{-}MA_{Y132F}$ was phosphorylated by the bacterially encoded tyrosine kinase, demonstrating that phosphorylation occurred specifically on Y132 of MA (upper panel, lanes 5 and 6). In addition, the last 14 residues of MA ($GST\text{-}MA_{119–132}$) conferred a level of terminal tyrosine phosphorylation equivalent to that of the full-length MA (lanes 4 and 5). And finally, the last five residues (GST-VSQNY) as well as the terminal tyrosine (GST-Y) were sufficient for phosphorylation, albeit at a lower level than GST-MA or $GST\text{-}MA_{119–132}$ (lanes 2 and 3). This difference in level of tyrosine phosphorylation was not due to a difference in amount of total protein loaded as confirmed by probing with an anti-GST antibody (lower panel).

MLV MA is C-terminally tyrosine phosphorylated. Because many retroviral MA proteins contain a tyrosine at their C termini due to the conservation of the tyrosine/proline cleavage site between MA and CA, it was of interest to determine if the MAs of HIV-1 and HIV-2 were unique in their C-terminal phosphorylation. We therefore asked whether the MA of MLV, a simple retrovirus, was similarly modified. An MLV MA_{Y131F} mutant in which the final tyrosine was replaced with a phenylalanine was generated in the context of an MLV Gag-Pol expression vector. This construct or its WT counterpart was cotransfected with a VSV-G envelope expression vector as well as a β-Gal-encoding MLV-based retroviral vector into 293T cells. The pseudotyped virions produced from the transfected cells were concentrated, normalized for RT activity, and subjected to Western blot analysis. Virions produced from cells expressing MA_{WT} of MLV contained a tyrosine-phosphorylated protein the size of MA that was not seen in the corresponding MA_{Y131F} virions (Fig. 6A, upper panel). This protein was subsequently confirmed to be MLV MA by probing the same blot with anti-RLV p15 antiserum (lower panel).

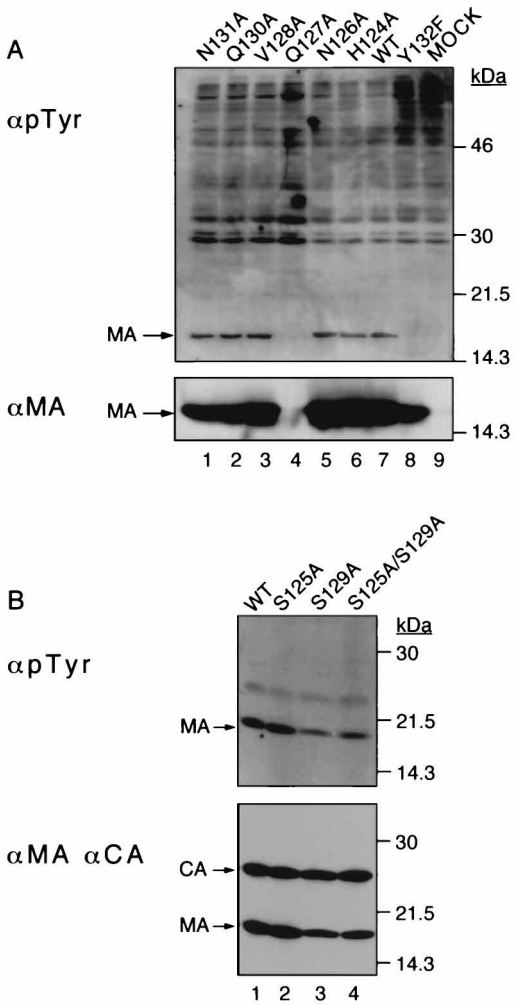

FIG. 4. Residues in the C terminus of MA are not critical for Y132 phosphorylation. (A) 293T cells were transfected with R7MASTOP mutant constructs (N131A, Q130A, V128A, Q127A, N126A, H124A, and Y132F) or the WT construct (WT) or were mock transfected (MOCK). Cells were lysed and subjected to Western blot analysis. Locations of molecular mass markers are shown on the right. (Upper panel) Lysates were probed with the anti-pTyr antiserum. (Lower panel) Same blot probed with an anti-MA antibody. (B) 293T cells were transfected with R7 (WT) or the MA mutant R7 constructs (S125A, S129A, and S125A/S129A). Cell-free supernatants were ultracentrifuged, normalized for p24 (CA) content (100 ng of p24), and analyzed by Western blotting. Molecular mass markers are shown on the right. (Upper panel) Virion proteins probed with anti-pTyr. (Lower panel) Subsequent probing of the blot with a mixture of anti-MA and anti-CA monoclonal antibodies to ensure that the pTyr-containing protein in the upper panel is MA and that equal amounts of virions were loaded.

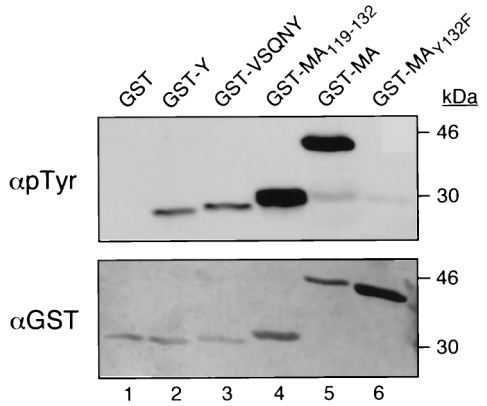

FIG. 5. A C-terminal tyrosine is sufficient for phosphorylation in vitro. TKX1 bacteria were transformed with pGEX-2T vectors encoding GST-MA fusion proteins. The fusion proteins were expressed and subjected to a tyrosine kinase activity in the bacteria. They were subsequently isolated on GST affinity columns and eluted with 10 mM glutathione. Normalized amounts of eluted protein (475 ng) were analyzed by Western blotting. (Upper panel) Fusion proteins were immunodetected with anti-pTyr antiserum. Lane 1, GST alone; lane 2, GST with a terminal tyrosine; lane 3, GST with the last five residues of HIV-1 MA; lane 4, GST with the last 14 residues of MA; lane 5, GST fused to full-length MA; lane 6, GST-MA with a terminal phenylalanine substitution. (Lower panel) Same blot subsequently probed with an anti-GST monoclonal antibody.

The phenylalanine substitution at the C terminus of MA did not appear to affect the stability or processing of Gag as there were equal levels of MA in the WT and Y131F virions. Thus, like HIV-1 MA, MLV MA is also phosphorylated on its C-terminal tyrosine. The infectivity of both the WT and Y131F-containing virions was assayed in rat 208F cells. No significant difference was observed between them in two separate experiments. For example, one experiment in which transfections and infections were done in triplicate yielded averages of 759 blue foci/1,000 cpm of RT for WT and 758 blue foci/1,000 cpm of RT for Y131F. Therefore, inhibition of MLV MA C-terminal tyrosine phosphorylation did not appear to attenuate viral infectivity in this system.

To ensure that the phosphorylation of MLV MA was not an artifact of its production in human 293T cells, we analyzed MLV virions produced from murine NIH 3T3 cells. Virions with WT MLV MA or with chimeric MA containing the first 128 amino acids of HIV-1 MA and the last 4 residues (SSLY) of MLV MA were probed with the anti-pTyr antibody. Both the WT and chimeric MA in NIH 3T3-produced virus were tyrosine phosphorylated (Fig. 6B, upper panel).

DISCUSSION

Employing an in vitro kinase assay, we have investigated the nature of the protein tyrosine kinase responsible for the phosphorylation of HIV-1 MA on its C-terminal tyrosine. Experiments revealed that the kinase activity is present in all cultured cell lines examined thus far. More relevantly, it is found in macrophages and activated PBLs, the natural targets of infection. Within the cell, the MA tyrosine kinase is found almost exclusively associated with membranes. Previously, we demonstrated that the MA tyrosine kinase activity is present in virions. Here we show that the kinase remains associated with the viral membrane and does not travel to the internal core of the virion with its phosphorylated substrate, MA. Thus, we propose a model whereby, during virion formation in producer cells, a membrane-associated protein tyrosine kinase is incorporated into budding virions. Following $Pr55^{Gag}$ cleavage, the kinase, which is in proximity to the MA molecules, phosphorylates a small subset of them. This phosphorylation allows them to associate with IN in the viral core and participate in the subsequent nuclear migration of the viral preintegration complex.

To date, the identity of the kinase remains elusive. Circumstantial evidence initially suggested that members of the Src family of nonreceptor tyrosine kinases might be candidates. First, Src family members are found in both macrophages and PBLs. Second, they are localized to the inner face of cell plasma membranes. In addition, recombinant murine Src is

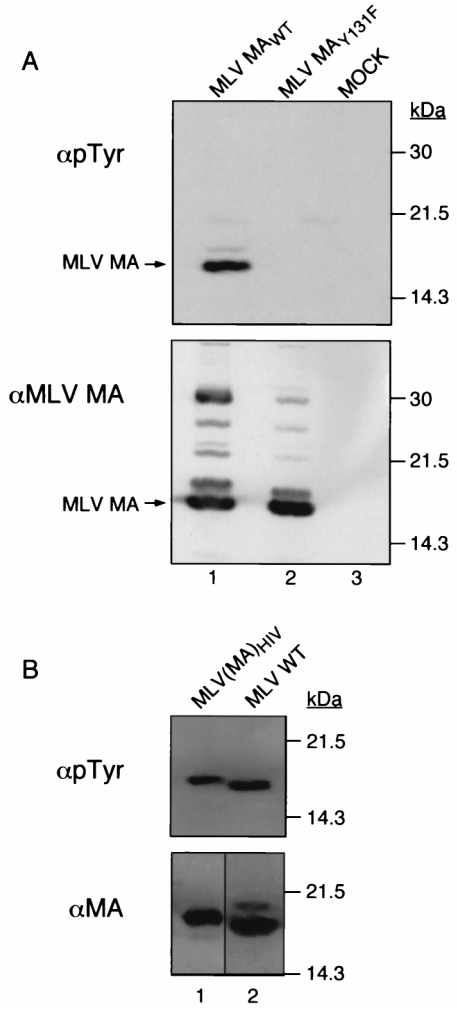

FIG. 6. MLV MA is phosphorylated on its C-terminal tyrosine. (A) 293T cells were cotransfected with pMD.G, pCLMFG-LacZ, and either pCMV-GAG POL (encoding MLV MA_{WT}) or pCMV-GAG(MA_{Y131F})POL (encoding MLV MA_{Y131F}). The resultant virions were normalized for RT activity and then analyzed by Western blotting. Molecular mass markers are shown on the right. (Upper panel) Viral lysates were probed with the anti-pTyr antiserum. Lane 1, MLV particles with WT MA; lane 2, MLV particles with Y131F mutant MA; lane 3, concentrated supernatant from mock-transfected cells. (Lower panel) The same blot probed with anti-RLV p15 antiserum to detect MLV MA to confirm that equal amounts of MLV MA were loaded in lanes 1 and 2. (B) Virus was isolated from NIH 3T3 cells constitutively expressing WT MLV or MLV with an HIV-1/MLV MA chimera. Virions were normalized for RT activity and subjected to Western blotting. (Upper panel) Viral lysates probed with anti-pTyr. Lane 1, chimeric HIV-1/MLV MA; lane 2, WT MLV MA. (Lower panel) Subsequent probing of the blot with anti-MA antibodies. Lane 1, detection of the chimeric MA with an anti-HIV-1 monoclonal antibody; lane 2, detection of MLV MA with the anti-RLV p15 antiserum.

capable of C-terminally phosphorylating MA in vitro. Another Src member which associates with CD4, Lck, when immunoprecipitated from CEM cells could also phosphorylate MA in vitro (43). And finally, Lck is found in virions (7). However, cell lines individually lacking Lck, Src, Fyn, and Yes were still able to phosphorylate MA at Y132 in the in vitro kinase assay (7). It remains a possibility, though, that any member of the family can act as an MA tyrosine kinase. Alternatively, it is possible that MA is phosphorylated by a receptor tyrosine kinase, consistent with our observation that GST-MA fusion proteins could be C-terminally tyrosine phosphorylated by a derivative of the Elk receptor tyrosine kinase expressed in bacteria.

The in vitro MA tyrosine kinase assay might not be best suited for identifying specific kinases due to its extreme sensitivity and the fact that, under the assay conditions, phosphorylation is favored. In addition, phosphorylation by a kinase in vitro does not guarantee that it will phosphorylate the same substrate in vivo. Therefore, while the in vitro assay provided a means for rapid screening of numerous cell lines and primary cells, a negative result might be more conclusive than a positive result. Nevertheless, HIV-1 virions produced from all cell lines tested to date including H9, CEM, SupT1, COS, 293, MT4, and Molt IIIB contain tyrosine-phosphorylated MA (1, 3, 19), a result that correlates well with the in vitro kinase assay. Furthermore, localization of MA tyrosine kinase activity to cell and viral membranes fits with our previous observation that MA needs to be membrane associated to be phosphorylated (19). Although we cannot definitively demonstrate that the tyrosine kinase which phosphorylates MA is one of the activities we see associated with viral membranes, it seems unlikely that it would be selectively excluded from virions while other kinase activities are not. In addition, we know that MA phosphorylation follows Gag cleavage (19) and therefore must occur in budding virions.

The occurrence of tyrosine as the C-terminal residue in proteins is roughly proportional to its frequency of usage (28). However, phosphorylation of a C-terminal tyrosine has been rarely reported. In fact, we know of only one cellular protein that undergoes such a modification, α-tubulin (46). We were therefore interested in determining the requirements for HIV-1 MA Y132 phosphorylation at the level of the substrate. Specifically, we questioned whether or not the eight residues (HSNQVSQN) immediately upstream of Y132 played a role in kinase recognition. Tyrosine kinases generally prefer acidic residues amino-terminal to the tyrosine substrate and are fairly selective for hydrophobic residues at the +3 position (41). However, at the time of phosphorylation, MA has no residues C-terminal to Y132. In addition, there are no glutamic or aspartic acids immediately upstream of Y132. Mutation of each of the eight residues preceding tyrosine did not affect Y132 phosphorylation, suggesting that the MA tyrosine kinase substrate specificity does not involve these residues. Confirming this lack of specificity, we observed that a virion-incorporated HIV-1/MLV MA chimera with the last four residues of MLV MA was capable of being phosphorylated. Additionally, GST with a C-terminal tyrosine (GST-Y) could be phosphorylated in vitro. However, the fact that GST-Y and GST-VSQNY were tyrosine phosphorylated much less efficiently than the GST-$MA_{119–132}$ fusion protein suggests that perhaps there is some element, either sequence or structure, between residues 119 and 128 of HIV-1 MA that is important for kinase recognition and activity. To probe this issue further, we created CD4 and HIV-1 Nef mutants which carried a C-terminal tyrosine. We failed to observe significant levels of C-terminal phosphorylation of these proteins upon expression in 293 cells (data not shown), indicating that not all membrane-associated proteins having tyrosine as their last residue become the target of kinases.

MLV	K P P P P L P P S A P S L P L E P P R S T P P R S S L Y
HIV-1_{HXB2}	E E E Q N K S K K K A Q Q A A A D T G H S N Q V S Q N Y
HIV-1_{NL4-3}	E E E Q N K S K K K A Q Q A A A D T G N N S Q V S Q N Y
HIV-2_{ROD}	L V A E T G T A E K M P S T S R P T A P S S E K G G N Y

FIG. 7. Comparison of amino acid sequences of MLV, HIV-1_{HXB2}, HIV-1_{NL4-3}, and HIV-2_{ROD} MA C termini.

The crystal structure of HIV-1 MA, which has been solved for residues 1 to 121, predicts that the first 104 amino acids form a single globular domain composed of five major helices capped by a mixed β-sheet. The last α-helix (amino acids 96 to 121) projects away from the packed bundle and makes the C-terminal 28 residues a distinct structural entity (25). MA is predicted to trimerize and membrane associate through its globular domain, while its C terminus projects away from the rest of the protein inside virions. Thus, it is possible that MA C-terminal tyrosine phosphorylation is simply a result of the accessibility of the C terminus to kinases that are localized to cell membranes rather than a specific kinase recognition and phosphorylation event. The fact that MLV MA is also C-terminally phosphorylated supports this possibility. Indeed, a comparison of the C-terminal sequences of MLV, HIV-1, and HIV-2 MA, which are all known to be phosphorylated on their terminal tyrosine residues, reveals little homology among the three proteins (Fig. 7) (33, 38).

MLV MA phosphorylation was initially a surprising finding. In the case of HIV-1, C-terminal tyrosine phosphorylation of MA has been demonstrated to facilitate infection of terminally differentiated macrophages in the absence of functional Vpr (19). MLV is known to be incapable of infecting nondividing cells and relies on nuclear envelope breakdown during mitosis in order to bring its viral nucleoprotein complex in contact with cell chromosomes (30, 36). Thus, the end result for MLV MA tyrosine phosphorylation cannot be the same as it is for HIV-1 MA. However, ability to infect nondividing cells is actually an indirect consequence of HIV-1 MA Y132 phosphorylation. The direct result is a pTyr-dependent association with HIV-1 IN. It is therefore possible that an analogous interaction occurs between tyrosine-phosphorylated MLV MA and MLV IN.

Future experiments should investigate the significance of MLV MA C-terminal tyrosine phosphorylation, in particular by determining the subviral localization of tyrosine-phosphorylated MLV MA as well as by testing its potential interaction with MLV IN. The functional significance of such a modification remains undetermined. Preliminary studies of VSV-G pseudotyped MLV retroviral vector particles containing MA_{Y131F} suggest that they are as infectious as those containing MA_{WT}. However, this assay system is somewhat artificial and thus may not readily reveal a phenotype for MA_{Y131F}. It will therefore be of interest to test this MA mutant within the context of replication-competent MLV in tissue culture as well as in vivo to determine which role MLV MA C-terminal tyrosine phosphorylation plays in the life cycle of this oncoretrovirus.

ACKNOWLEDGMENTS

We thank L. Barden for the artwork and our colleagues for helpful discussions.

This work was supported by a grant from the University of California Universitywide AIDS Research Program (Award R95-SAL-111) and by a

Public Health Service Award (AI37510) to D.T. D.C. is a member of the joint University of California San Diego, Biology Department/Salk Institute graduate program.

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