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HIV Nuclear Import Is Governed by the Phosphotyrosine-Mediated Binding of Matrix to the Core Domain of Integrase

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Summary

The karyophilic properties of the viral matrix (MA) protein govern HIV nuclear import in nondividing cells such as macrophages. A critical regulator of this process is the C-terminal tyrosine phosphorylation of MA during virus maturation. Here, we reveal the mechanism of this phenomenon, by demonstrating that tyrosine phosphorylation induces the binding of MA to integrase (IN). This leads to the incorporation of MA molecules into virus cores, and subsequently into uncoated viral nucleoprotein complexes. A direct interaction between tyrosine-phosphorylated MA and the central domain of IN can be demonstrated in vitro. It is blocked by phosphotyrosine, indicating that IN recognizes the phosphorylated C-terminal residue of MA. These results explain how the karyophilic potential of MA is conferred to the HIV nucleoprotein complex.

Introduction

Human immunodeficiency virus (HIV) replication in nonmitotic cells such as terminally differentiated macrophages is likely crucial for its transmission and for its spread and persistence in the body, as well as for AIDS induction (Meltzer and Gendelman, 1992). In contrast with oncoretroviruses (Humphries and Temin, 1972, 1974; Roe et al., 1993; Lewis and Emerman, 1994), HIV can infect nonproliferating targets, because it encodes determinants that govern the active transport of the virus nucleoprotein complex through the nucleopore, allowing integration into the host cell chromosome in the absence of mitosis (Weinberg et al., 1991; Bukrinsky et al., 1992; Lewis et al., 1992).

Two viral proteins, the viral matrix protein (MA) and Vpr, mediate this process (Bukrinsky et al., 1993; Heinzinger et al., 1994; von Schwedler et al., 1994). MA, the myristoylated N-terminal cleavage product of the HIV-1 p55 Gag precursor, has intrinsic karyophilic properties conferred by a conserved stretch of basic residues, which acts as a nuclear localization signal (NLS) (Bukrinsky et al., 1993). In the absence of a functional *vpr* gene, MA-NLS mutant viruses fail to replicate efficiently in macrophages, owing to a block in nuclear import (Bukrinsky et al., 1993; Heinzinger et al., 1994; von Schwedler et al., 1994). As predicted from this result, MA can be detected in the nucleus of newly infected cells during the early steps of the replicative cycle (Sharova and Bukrinskaya, 1991). In contrast, in virus-producing cells, MA localizes to the plasma membrane, owing to the combined effects of myristoylation and of positively charged residues near the protein N-terminus (Yuan et al., 1993; Spearman et al., 1994; Zhou et al., 1994). These latter residues are thought to establish electrostatic interactions with the negatively charged phospholipids on the inner leaflet of the lipid bilayer (Zhou et al., 1994). The membrane association of MA is essential for both the proper assembly of virions and their efficient release from cells (Varmus and Swanstrom, 1984).

The myristoylation signal and the NLS thus exert conflicting influences on the subcellular localization of MA. We recently demonstrated that a key to the sequential action of these motifs is the phosphorylation of a portion of MA molecules on the C-terminal tyrosine at the time of virus maturation, by a virion-associated cellular tyrosine kinase (Gallay et al., 1995). After viral entry, tyrosine-phosphorylated MA molecules are preferentially transported to the nucleus, whereas the bulk of MA, not phosphorylated on tyrosine, stays at the plasma membrane. Replacing the C-terminal tyrosine of MA by a phenylalanine does not impair HIV-1 replication in dividing cells, but the resulting virus is markedly defective for growth in terminally differentiated macrophages, owing to a block in nuclear import.

MA tyrosine phosphorylation does not increase the karyophilic potential of MA per se, because nonmyristoylated MA is effectively translocated to the nucleus even though it is not tyrosine phosphorylated (Gallay et al., 1995). It is therefore more likely that tyrosine phosphorylation triggers the redistribution of MA from the membrane toward the inner regions of the virus, thereby allowing the NLS to play its role during the early steps of infection. This could be achieved by disrupting electrostatic interactions between MA and the membrane, analogous to what is thought to account for the phosphorylation-induced cytosolic release of MARCKS and $pp^{60c\text{-}src}$ (Thelen et al., 1991; Walker et al., 1993). Alternatively, albeit not exclusively, phosphorylation could stimulate the binding of MA to another component of the viral nucleoprotein core. Here, we present evidences supporting this second mechanism.

Results

Tyrosine-Phosphorylated MA Is Found in the Virion Core

The subviral distribution of tyrosine-phosphorylated MA was examined by partial lysis of virions followed by sucrose gradient fractionation, which separates viral cores and membranes. HIV-2 was used for this experiment, because HIV-1 particles were found to be too unstable in the presence of detergent. Fractions were analyzed for their content of reverse transcriptase (RT), p27 capsid (CA), integrase (IN), envelope (Env), MA, and tyrosine-phosphorylated MA through a combination of enzymatic and immunological assays (Figure 1A). Intact HIV-2 virions exhibited a density of 1.15 to 1.19 g/cm^3, as indicated by

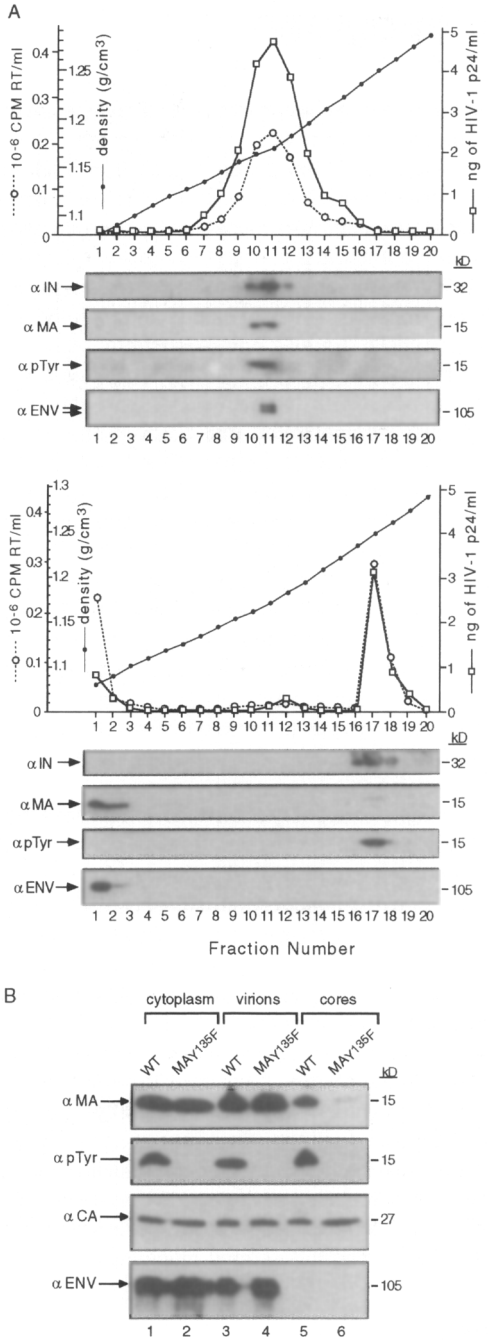

Figure 1. Phosphotyrosine-Dependent Incorporation of MA into the Virion Core

(A) Purified HIV-2 virions were fractionated on a sucrose gradient, before (top) and after (bottom) partial lysis. Fractions were evaluated for their content in RT activity (by an exogenous RT assay) and p27 CA antigen (using the HIV-1 p24 ELISA), as well as for the presence of IN, MA, tyrosine-phosphorylated MA, and Env (by Western blot). The p24 ELISA is approximately 1000–5000 times less sensitive for the HIV-2 CA than for its HIV-1 counterpart, so that the numbers shown here only represent relative values.

(B) Cytoplasmic extracts and virions released from CEM cells infected with wild-type or tyrosine-mutated HIV-2, as well as purified viral cores, were analyzed by Western blot with the indicated antibodies, with (pTyr, CA, Env) or without (MA) prior immunoprecipitation.

the recovery of all viral proteins in the corresponding fractions of the gradient (Figure 1A, top). After the particles had been treated with a mild detergent, a sharp peak of RT activity was detected at a density of 1.24–1.25 g/cm^3 (Figure 1A, bottom). This peak also contained CA and IN, but no Env, indicating that it likely corresponded to viral cores. Most MA molecules were retained at the top of the gradient, together with Env, consistent with the membrane association of these two proteins. In contrast, all tyrosine-phosphorylated MA molecules were detected in the fractions corresponding to viral cores.

A mutant HIV-2 clone was generated, in which the C-terminal tyrosine of MA was replaced by a phenylalanine. The resulting virus, named $MA_{Y135F}HIV\text{-}2_{ROD}$, grew with normal kinetics in proliferating T cells (data not shown). Cytoplasmic extracts and virions released from CEM cells infected with wild-type or tyrosine-mutated HIV-2 were compared for their content in total and tyrosine-phosphorylated MA (Figure 1B, lanes 1–4). The MA_{Y135F} mutation abolished the reactivity of MA with a phosphotyrosine-specific antibody, indicating that HIV-2 MA, like its HIV-1 counterpart, undergoes phosphorylation on its C-terminal tyrosine. Viral cores prepared from wild-type and mutant HIV-2 were then examined (Figure 1B, lanes 5 and 6). Whereas the mutation did not affect the subviral distribution of CA and Env, MA levels in cores of $MA_{Y135F}HIV\text{-}2_{ROD}$ were greatly reduced compared with those in the wild-type control.

Tyrosine-Phosphorylated MA Is Associated with the HIV-1 Nucleoprotein Complex

A prediction derived from this and our previous experiments was that tyrosine-phosphorylated MA molecules might be selectively incorporated into uncoated viral nucleoprotein complexes, following fusion of the virus and target cell membranes. To address this point, cytoplasmic extracts of T cells freshly infected with HIV-1 were fractionated on a sucrose gradient. Fractions were analyzed for their content in viral proteins and DNA by Western blot and polymerase chain reaction (PCR), respectively (Figure 2). The p24 CA antigen as well as the bulk of MA were detected in the region of the gradient with the lowest density, indicating that these proteins were dissociated from the viral nucleoprotein complex. In contrast, a peak of RT activity was detected at a density of 1.21–1.25 g/cm^3, together with high levels of IN, Vpr, and nucleocapsid (NC), and the viral DNA. These fractions, consistent with partially purified viral nucleoprotein complexes, also contained all tyrosine-phosphorylated MA molecules, even though this subspecies only represented a small fraction of the total amount of MA.

To examine further the subcellular distribution of various proteins during the early steps of the virus life cycle, cells were acutely infected with either wild-type HIV-1 or mutant MA_{Y132F}, in which the C-terminal tyrosine of MA is changed to phenylalanine (Gallay et al., 1995). At 1 hr and 8 hr postinfection, cells were fractionated into membranes, cytosol, and nucleus. Fractions were analyzed by immunoprecipitation and Western blot with antibodies against MA, phosphotyrosine, IN, Vpr, NC, and Env, as well as by measuring RT activity and p24 antigen content (Figure 3). At

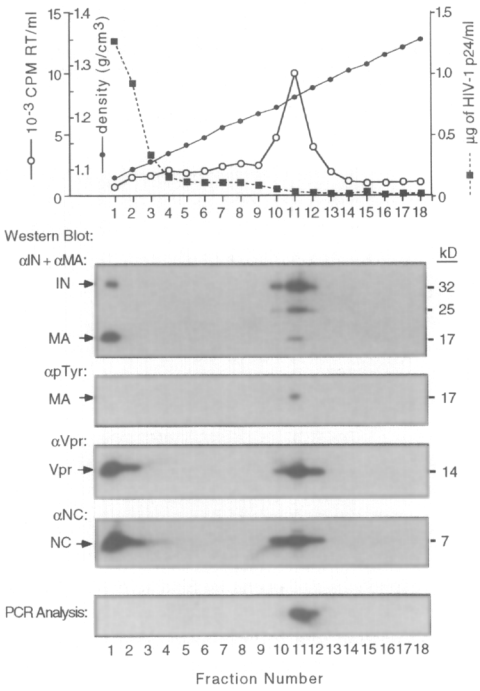

Figure 2. Tyrosine-Phosphorylated MA Associates with the Virus Nucleoprotein Complex

Cytoplasmic extracts of SupT1 cells acutely infected with HIV-1 were fractionated on a sucrose gradient, and fractions were evaluated first for the presence of various viral proteins by a combination of immunoprecipitations and Western blots (for Vpr a simple Western blot was used) and second for their content in viral DNA by PCR.

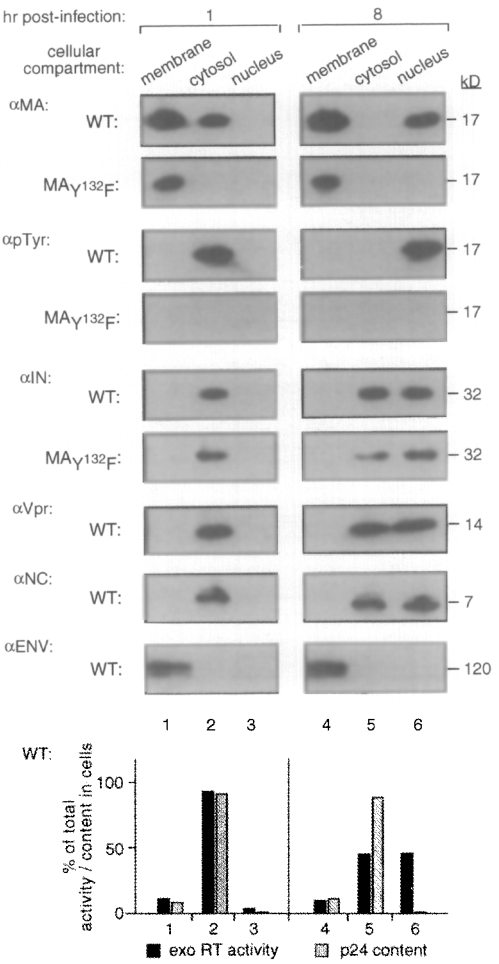

Figure 3. Subcellular Localization of Viral Proteins during the Early Steps of Infection

Extracts of P4-2 cells acutely infected with wild-type HIV-1, or with the tyrosine-mutated MA_{Y132F} variant, were fractionated at 1 and 8 hr postinfection in membranes, cytosol, and nucleus. The presence of various viral proteins was monitored by immunoprecipitation followed by Western blot with the indicated antibodies (top; for Vpr, a simple Western blot was used), or by measuring RT activity and p24 CA antigen content (bottom graph). The subcellular distributions of NC, Vpr, Env, p24, and RT in MA_{Y132F}-infected cells were identical to those observed for wild type (data not shown).

1 hr postinfection, in cells infected with either virus, Env was found in membranes (lane 1), whereas IN, Vpr, NC, p24, and RT were detected in the cytosol (lane 2; data not shown). In wild-type infected cells, although MA was associated predominantly with membranes (lane 1), some of this protein, and most importantly all of its tyrosine-phosphorylated component, was present in the cytosol (lane 2). In contrast, in MA_{Y132F}-infected cells, no MA was detected in the cytosol (lane 2). At 8 hr postinfection, in cells infected with either virus, approximately half of IN, Vpr, NC, and RT had migrated to the nucleus (lane 6), whereas CA remained in the cytosol (lane 5). Although tyrosine-phosphorylated MA was found exclusively in the nucleus of wild-type infected cells (lane 6), MA was restricted to the membrane fraction of cells inoculated with the MA_{Y132F} variant (lane 4). These data thus confirm that the C-terminal tyrosine of MA is necessary for its nuclear migration.

Tyrosine-Phosphorylated MA Is Associated with Integrase In Vivo

The possible association of tyrosine-phosphorylated MA with other viral proteins was examined by coimmunoprecipitation (Figure 4). In partially purified viral nucleoprotein complexes, MA- and phosphotyrosine-specific antibodies could immunoprecipitate IN (Figure 4A), but failed to precipitate either NC, Vpr, or RT (data not shown). Reciprocally, immunoprecipitations with IN-specific antibodies selectively depleted MA from viral nucleoprotein complexes (data not shown). In contrast, in cells infected with the MA_{Y132F} virus, no association between IN and MA was detected (data not shown), corroborating the distinct subcellular localizations of these two proteins (see Figure 3).

IN- and phosphotyrosine-specific antibodies could also immunoprecipitate all MA molecules contained in purified HIV-2 cores (Figure 4B), but not RT or p27 CA (data not shown). This indicates that the association between MA and IN, already established in virions, does not require structural modifications occurring after viral entry, nor cellular factors that might act on the virus during uncoating.

These results raised the possibility that MA translocation in target cells might need the binding of this protein to integrase. To test this hypothesis, an HIV-1 mutant lacking

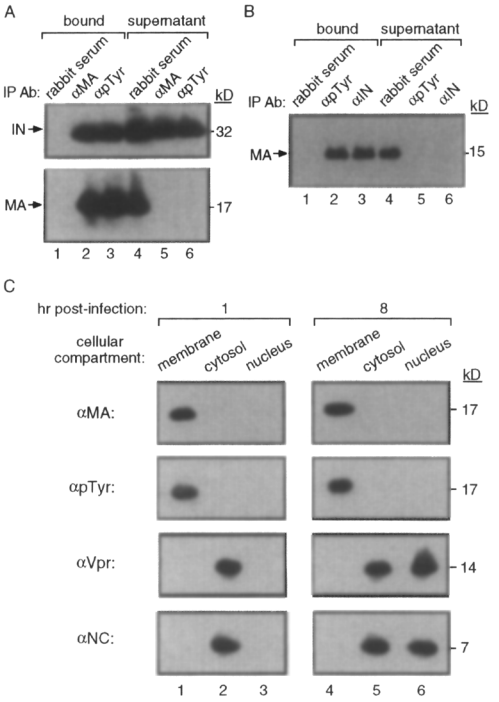

Figure 4. MA Associates with the HIV Nucleoprotein Complex through an Interaction with Integrase

(A) HIV-1 nucleoprotein complexes partially purified from the cytoplasm of acutely infected SupT1 cells were immunoprecipitated with the antibodies shown on top of each lane. Bound material and supernatant were analyzed by Western blot with indicated antiserums.

(B) Same experiment, on purified HIV-2 cores. RT activity and p27 CA antigen were not coimmunoprecipitated by the various antibodies (data not shown).

(C) MA subcellular localization in cells infected with IN-defective HIV-1, analyzed as described in Figure 3.

integrase, called ΔIN, was generated by introducing a stop codon at the 3' end of the RT coding sequence. This modification had no effect on the production of viral particles (data not shown). Furthermore, the translocation of NC and Vpr was as efficient in ΔIN- as in wild type–infected cells (compare Figure 4C with Figure 3). In contrast, MA was retained at the membrane of cells inoculated with the integrase-defective virus. The presence of IN is thus required for the cytosolic and nuclear translocation of MA during the early steps of HIV infection.

Phosphotyrosine-Mediated Binding of MA to the Core Domain of Integrase In Vitro

The data presented so far would not allow one to conclude that MA and integrase interact directly. This question was addressed in vitro by using Escherichia coli–produced recombinant molecules (Figure 5). Two forms of HIV-1 MA were tested: the wild-type protein and the MA_{Y132F} mutant. The effect of tyrosine phosphorylation on MA–IN binding was further assessed by treating the two MA variants with Src kinase. In vitro, Src specifically phosphorylates the C-terminal tyrosine of MA, as demonstrated by phosphoamino acid analysis and tryptic peptide mapping of Src-phosphorylated wild-type MA, as well as by the failure of

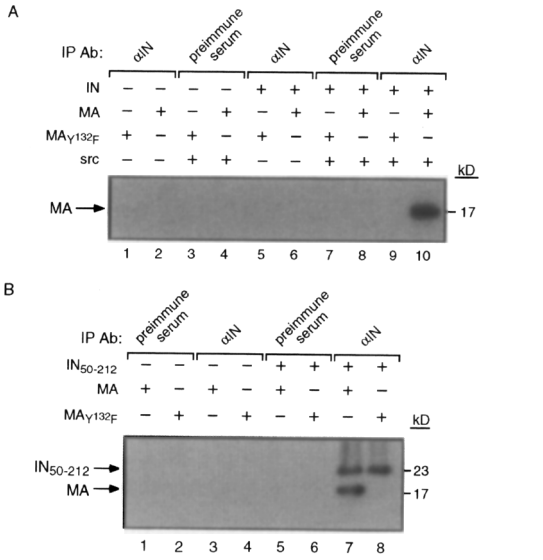

Figure 5. Phosphotyrosine-Dependent Binding of MA to Integrase In Vitro

(A) Recombinant wild-type or tyrosine-mutated MA proteins were mixed with integrase, with and without prior treatment with Src kinase, as indicated, with [^{32}P]γATP as phosphate donor. Products were immunoprecipitated with control or IN-specific antibody, and analyzed by SDS–PAGE and autoradiography.

(B) Same experiment, using Src-treated MA variants and a recombinant molecule corresponding to amino acids 50–212 of integrase. Immunoprecipitations were performed with IN-specific antibody, and detection by Western blot with antiserums against MA and IN.

this kinase to induce the incorporation of phosphate into the MA_{Y132F} mutant (data not shown). Wild-type and mutant forms of MA were mixed with recombinant HIV-1 IN, with or without prior phosphorylation with Src. The formation of MA–IN complexes was monitored by immunoprecipitation with IN-specific or control antibodies, followed by gel electrophoresis and either autoradiography (if radiolabeled phosphate had been used in the kinase reaction) (Figure 5A) or Western blot with IN- and MA-specific antiserums (Figure 5B). No association of IN with MA_{Y132F} or with the nonphosphorylated form of wild-type MA was observed (Figure 5A, lanes 7–9). In contrast, the tyrosine phosphorylation of MA promoted the formation of a complex with IN (Figure 5A, lane 10). Depleting Src-treated MA with a phosphotyrosine-specific antibody prevented the formation of MA–IN complexes (data not shown). Therefore, the interaction between MA and IN is tyrosine phosphorylation–dependent and requires neither additional viral proteins nor the viral genome.

To explore further the requirements for the interaction between IN and tyrosine-phosphorylated MA, a truncated version of integrase corresponding to the minimal catalytically active core domain of this enzyme, from amino acid 50 to amino acid 212 (Bushman et al., 1993; Vink et al., 1993), was tested for its ability to bind phosphotyrosine-containing MA (Figure 5B). As observed with the full-length molecule, IN_{50-212} associated with the tyrosine-phosphorylated form of wild-type MA, but failed to bind to the MA_{Y132F} variant (Figure 5B, lanes 7 and 8).

That tyrosine phosphorylation of MA was required for IN binding did not prove that the modified C-terminal residue of MA was itself involved in mediating the interaction between the two proteins. Instead, it could have been that this modification induced a conformational change in MA that resulted in exposing a distantly placed motif responsible for IN recognition. To address this question, three experiments were performed.

First, the resistance of tyrosine-phosphorylated MA to phosphatase was evaluated, before and after formation of a complex with integrase (Figure 6A). Calf intestinal phosphatase efficiently removed the phosphate from tyrosine-phosphorylated MA (lane 2) and as a consequence prevented the formation of MA–IN complexes (lane 5). In contrast, when preformed MA–IN complexes were exposed to phosphatase, tyrosine-phosphorylated MA was resistant to dephosphorylation (lanes 3 and 6), and the treatment did not disrupt the association between the two proteins (lane 6). This indicates that the C-terminal phosphotyrosine of MA is protected from the action of phosphatase following integrase binding.

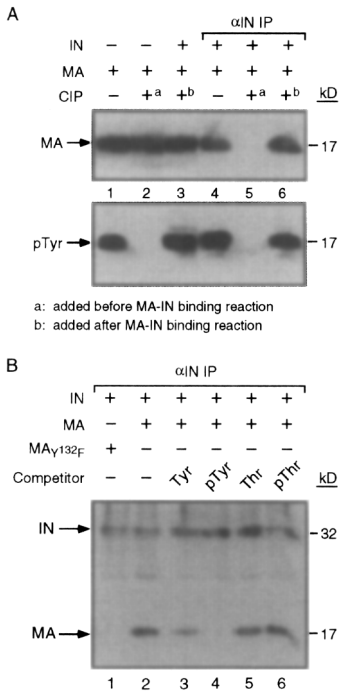

Figure 6. The C-terminal Phosphotyrosine of MA Is Involved in Mediating MA–IN Complex Formation

(A) Calf intestine phosphatase was added to Src-treated MA as indicated, before (lanes 2 and 5) or after (lanes 3 and 6) performing an MA–IN-binding reaction. Products were separated by SDS–PAGE, either directly (lanes 1 to 3) or after immunoprecipitation with anti-IN antibody; results were analyzed by Western blot with antibodies against MA (top) or phosphotyrosine (bottom).

(B) Src-treated wild-type and tyrosine-mutated versions of recombinant MA were mixed with recombinant integrase preincubated with various competitors as indicated. Products were immunoprecipitated with IN-specific antibody and analyzed by Western blot with a mixture of antibodies against MA and integrase.

Second, the ability of various amino acids to compete for this reaction was evaluated (Figure 6B). Preincubating integrase with tyrosine, threonine, or phosphothreonine did not interfere with the association of this protein with MA (compare lane 2 with lanes 3, 5, and 6), nor did serine or phosphoserine (data not shown). In contrast, phosphotyrosine completely blocked the interaction between the two molecules (lane 4).

Finally, preincubating tyrosine-phosphorylated MA with an excess of anti-phosphotyrosine antibody prevented the formation of MA–IN complexes (data not shown). On the basis of these experiments, it can be concluded that a phosphotyrosine-binding site in the core domain of integrase is responsible for recognizing the phosphorylated C-terminus of MA directly.

Discussion

This study reveals how the karyophilic properties of MA are conferred to the HIV nucleoprotein complex, thereby governing virus nuclear import in nondividing cells. MA carries two motifs, a myristoylation signal and an NLS, that exert conflicting influences on its subcellular localization. In virus producer cells, the protein associates with the plasma membrane as part of the Gag precursor by virtue of its N-terminal myristate. A subset of MA molecules is then phosphorylated on C-terminal tyrosine during virion maturation. This modification induces the formation of a complex with integrase, triggering the redistribution of some MA to the inner regions of the particle. After fusion of the virus and target cell membranes, tyrosine-phosphorylated MA, still associated with integrase, becomes part of the uncoated viral nucleoprotein complex, together with the viral genome, RT, NC, and Vpr. It can then exert its karyophilic role, most probably through the NLS-mediated recognition of a cytoplasmic receptor that directs the HIV nucleoprotein complex to the nucleopore.

Tyrosine phosphorylation enhances the incorporation of MA into the virus core and is required for its association with the uncoated viral nucleoprotein complex. Furthermore, all MA molecules present in cores and in partially purified nucleoprotein complexes can be immunoprecipitated with a phosphotyrosine antibody, at least in the presence of low concentrations of detergent. Nevertheless, in the presence of high concentrations of detergent, not all MA molecules contained in the HIV-1 nucleoprotein complex are immunoprecipitated by the anti-phosphotyrosine antibody (data not shown). Although dephosphorylation of the protein during preparation of the extracts cannot be formally excluded, it suggests that MA exists as a mixed multimer, comprising both tyrosine-phosphorylated and nontyrosine-phosphorylated subunits. The recently obtained crystal structure of MA supports this hypothesis, as it reveals that MA assembles as a close-packed oligomer (C. Hill and W. Sunquist, personal communication). Notably, a subpopulation of virion-associated MA is phosphorylated on several serines, but not on tyrosine (S. S. and D. T., unpublished data). It will be interesting to determine whether these serine-phosphorylated MA molecules

are the ones that are incorporated in cores and in uncoated nucleoprotein complexes, together with tyrosine-phosphorylated MA.

Three lines of evidence suggest that the modified C-terminus of MA is itself involved in contacting integrase. First, the covalent bond between phosphate and the C-terminal tyrosine of MA becomes resistant to the action of phosphatase once MA associates with integrase. Second, preincubation of integrase with phosphotyrosine efficiently prevents MA binding, whereas tyrosine, threonine, serine, phosphothreonine, and phosphoserine have no such effect. Finally, preincubation of MA with anti-phosphotyrosine antibodies inhibits MA–IN complex formation. The central region of integrase must therefore contain a motif capable of recognizing phosphotyrosine, either as a free amino acid or as the C-terminal residue of MA. The immunoprecipitation of MA–IN complexes with anti-phosphotyrosine antibodies may seem incompatible with this model. However, this paradox is likely a consequence of MA multimerization. Of note, the phosphotyrosine-specific antibody did not immunoprecipitate MA–IN complexes formed in vitro (data not shown), suggesting that in that case MA did not oligomerize.

Prior to MA, we had not seen a protein phosphorylated on a C-terminal tyrosine. It is therefore not surprising to find no similarity between the sequence of the IN core and that of previously described phosphotyrosine-binding domains, such as Src homology domain 2 (Pawson and Schlessinger, 1993), or the recently described PID (phosphotyrosine interaction domain) (Blaikie et al., 1994; Kavanaugh and Williams, 1994; Bork and Margolis, 1995). A critical step of the HIV life cycle thus appears to be mediated by a novel type of protein–protein interaction.

Experimental Procedures

DNA Constructions

Plasmid R8, containing a full-length HIV-1 proviral DNA in which all reading frames are functional, was obtained by cloning a BssHII–BamHI fragment from HIV-1_{NL4-3} (Myers et al., 1992) into the previously described R7 construct (Kim et al., 1989). Plasmid HIV-2_{ROD10}, a gift from M. Emerman, expresses the HIV-2_{ROD} proviral DNA. PCR-mediated mutagenesis was used to substitute a phenylalanine for the C-terminal tyrosine of MA in R8 and in HIV-2_{ROD10}, and to introduce a stop codon at the 3' end of the RT coding sequence. Recombinant HIV-1 MA carrying an N-terminal histidine tag was produced in E. coli by using the bacterial expression vector pET-15b (Novagen) and purified by affinity chromatography on a nickel–Sepharose column according to the instructions of the manufacturer. Full-length and truncated versions of recombinant integrase were generated as previously described (Bushman et al., 1993).

Cells

Cell lines were grown as previously described (Aiken et al., 1994). HIV-1-infected Molt IIIB human T lymphoid cells (Farnet and Haseltine, 1991) were a gift from C. Farnet, Salk Institute, and CD4-positive HeLa-derived P4-2 cells (Charneau et al., 1994) were a gift from F. Clavel, Pasteur Institute.

Transfections and Infections

Transfections and infections were performed as previously described (Aiken et al., 1994; Gallay et al., 1995). Viral stocks of R8 and HIV-2 derivatives were produced either by transfection of human fibroblastic 293 cells, or by electroporation of CEM cells. SupT1 cells were infected either by coculture with Molt IIIB cells at a ratio of 50:1 (2×10^7 Molt IIIB cells per 10^9 SupT1 cells) or by cell-free virus infection (80 μg of p24 antigen per 10^9 SupT1 cells). P4-2 cells were infected by the addition of cell-free virus (50 μg of p24 antigen per 10^8 P4-2 cells). At various times postinfection, cells were washed three times with PBS, trypsinized, and washed again.

Core and Nucleoprotein Complex Purification

Purification of HIV-2 cores was performed according to a technique optimized by V. Kewalramani and M. Emerman. In brief, 600 ml of supernatant from CEM cells acutely infected with HIV-2_{ROD} was filtered through a 0.45 μm (pore size) nitrocellulose membrane and centrifuged through a 20% sucrose cushion at 23,000 rpm in an SW28 rotor for 2 hr. Pelleted virions were resuspended in 500 μl of PBS containing 1% Igepal CO-630 (Rhone–Poulenc) and immediately overlayed onto a 20%–60% sucrose gradient. After 24 hr at 20,000 rpm in an SW40 rotor, fractions were collected and tested for their content in various viral proteins. For MA–IN coimmunoprecipitation studies, cores were further treated with 0.0025% Brij-96 (Sigma). HIV-1 viral nucleoprotein complexes were purified from SupT1 cells acutely infected with IIIB viruses at 5 hr postinfection as previously described, with 0.025% Brij-96 as detergent (Miller et al., 1995). Subcellular fractionation of acutely infected P4-2 cells was performed as recently described (Gallay et al., 1995).

Detection of Viral Proteins

HIV-1 p24 antigen values in virus and cell extracts were measured by enzyme-linked immunosorbent assay (ELISA) (DuPont). The CA content of HIV-2 virions and cores was determined by using the same assay. This ELISA is approximately 1000–5000 times less sensitive for the HIV-2 CA than for its HIV-1 counterpart, so that the numbers shown in Figure 1 only represent relative values. RT activity was monitored by a so-called exogenous RT assay (Goff et al., 1981), with minor modifications (Aiken and Trono, 1995). Other viral proteins were detected by Western blot with specific antibodies, with or without prior immunoprecipitation, as previously described (Gallay et al., 1995). Polyclonal serums against HIV-1 MA and IN were obtained by immunizing rabbits with E. coli-produced recombinant proteins. Both antisera cross-react with their respective HIV-2 counterparts, although the anti-HIV-1 MA antibody fails to immunoprecipitate HIV-2 MA. Rabbit anti-phosphotyrosine serum was a gift from B. Sefton, Salk Institute. Rabbit anti-HIV-1 Vpr was a gift from L. Ratner, obtained through the National Institutes of Health AIDS Research and Reference Program. Rabbit anti-NC serum was a gift of L. Henderson, National Cancer Institute–FCRDC. Monoclonal antibodies against HIV-1 MA, HIV-1 RT, and HIV-2 CA were purchased from Advanced Biotechnologies, Incorporated, and monoclonal antibodies specific for HIV-2 MA, HIV-1 Env, and HIV-2 Env were acquired from Biotech Research Laboratories, Genzyme, and Intracel, respectively.

MA-Integrase In Vitro Binding Assays

Recombinant histidine-tagged MA was tyrosine phosphorylated in vitro with recombinant mouse Src expressed in SF9 insect cells (a gift from M. Broome and T. Hunter, Salk Institute). Kinase reactions were conducted for 2 hr at 37°C, using 20 ng of Src for 5 μg of MA in 30 μl of a solution containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 50 mM NaCl, 1 mM orthovanadate, and 0.1% NP-40, 5 mM cold ATP, with or without 30 μCi of [^{32}P]γATP. The product was purified by affinity chromatography on nickel–Sepharose beads, as previously described (Gallay et al., 1995). MA (100 ng) was then incubated with recombinant full-length IN (100 ng), or with its truncated version, IN_{50-212}, for 1 hr at 37°C in 1 ml of binding buffer (20 mM HEPES [pH 7.3], 150 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.25% Brij-96). Complexes were immunoprecipitated by using anti-IN antibodies covalently coupled to CNBr-activated agarose for 2 hr at 4°C. After ten washes with binding buffer supplemented with 0.25 M NaCl, immunoprecipitated proteins were eluted from the beads with SDS–loading buffer and loaded onto a 10% SDS–polyacrylamide gel. Proteins were then transferred to PVDF membrane by electroblotting and analyzed by Western blotting with anti-IN, anti-MA, or anti-phosphotyrosine antibodies. Calf intestinal phosphatase (2 U) was used to treat 100 ng of MA for 2 hr at 37°C. In competition experiments, 100 ng of IN was

preincubated with 1 mM of free amino acids for 1 hr at 37°C, in 1 ml of binding buffer (pH 7.3), without detergent.

PCR Analysis of Acutely Infected Cells

PCR analysis was performed as previously described (Trono, 1992). The sequences of HIV-specific primers are as follows (positions of nucleotides in the HIV-1_{HXB2} sequence, according to Myers et al. [1992], are indicated in parentheses). Vif 6, GGGAAAGCTAGGGGATGGTTT-TAT (5136 to 5159); Vif 7, CAGGGTCTACTTGTGTGCTATTC (5340 to 5317). Vif 6 and Vif 7 amplify elongated minus-strand DNA.

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