Identification of Three Major Phosphorylation Sites within HIV-1 Capsid

ROLE OF PHOSPHORYLATION DURING THE EARLY STEPS OF INFECTION*

(Received for publication, December 28, 1998, and in revised form, April 12, 1999)

Christine Cartier‡§, Peggy Sivard‡, Corinne Tranchat‡, Didier Decimo‡, Claude Desgranges‡, and Véronique Boyer¶

From ‡Virus des Hepatites, Rétrovirus Humains et Pathologies Associées, INSERM U271, 151 Cours. A. Thomas, 69 424 Lyon Cedex 03, France and the ¶LaboRetro, Unité de Virologie Humaine, INSERM U412, École Normale Supérieure de Lyon, 46 allée d’Italie, 69 364 Lyon Cedex 07, France

We previously reported the presence of two cellular serine/threonine protein kinases incorporated in human immunodeficiency virus type 1 (HIV-1) particles. One protein kinase is MAPK ERK2 (mitogen-activated protein kinase), whereas the other one, a 53-kDa protein, still needs to be identified. Furthermore, we demonstrated that the capsid protein CAp24 is phosphorylated by one of those two virion-associated protein kinases (Cartier, C., Deckert, M., Grangeasse, C., Trauger, R., Jensen, F., Bernard, A., Cozzone, A., Desgranges, C., and Boyer, V. (1997) J. Virol. 71, 4832–4837). In this study, we showed that CAp24 is not a direct substrate of MAPK ERK2. Moreover, using site-directed mutagenesis of each of the 9 serine residues of CAp24, we demonstrated the phosphorylation of 3 serine residues (Ser-109, Ser-149, and Ser-178) in the CAp24. Substitution of each serine residue did not affect viral budding, nor viral structure. By contrast, substitution of Ser-109, Ser-149, or Ser-178 affects viral infectivity by preventing the reverse transcription process to be completely achieved. Our results suggest that CAp24 serine phosphorylation is essential for viral uncoating process.

The retroviral gag gene encodes the major structural proteins of HIV-1 particles. The p55\textsuperscript{core} precursor is cleaved by the viral protease in different proteins: the matrix MAp17, the capsid CAp24, the p6\textsuperscript{core}, the nucleocapsid NCp7 associated with viral RNA, and finally, two small spacer peptides p2 and p1 (1). The Gag protein expression in a cellular context is with viral RNA, and finally, two small spacer peptides p2 and p1 (1). The Gag protein expression in a cellular context is sufficient to generate retroviral-like particles that bud at the plasma membrane (for review, see Ref. 2). Moreover, Gag proteins are able to produce immature capsids that lack a complete lipid envelope in a cell-free reaction (3). However, although proteolytic processing is not a prerequisite for the formation of particles, it is absolutely essential for infectivity (4).

Phosphorylation of viral proteins plays an important role in regulation of viral cycle (for review, see Ref. 5). Several HIV-1 proteins, including structural and accessory proteins, have been shown to be phosphorylated. The cytoplasmic domain of Vpu is phosphorylated by casein kinase 2 at 2 serine residues, and this phosphorylation is necessary for the degradation of CD4 in endoplasmic reticulum (6–9). Nef is phosphorylated by the protein kinase C in vitro (10–12) and in vivo (13), and this phosphorylation leads to an increased down-regulation of CD4 from the cell surface (14). Rev is phosphorylated in vitro by casein kinase 2 and mitogen-activated protein kinase (MAPK) (15–17), promoting rapid formation of an efficient RNA binding state (18). Vif is phosphorylated in vitro and in vivo on 5 serine residues, 2 of those are phosphorylated by MAPK and the 3 others by another unidentified protein kinase. The phosphorylation of at least two of those sites seems to be essential for Vif function and HIV-1 replication (19, 20). Two forms of Tat are synthesized during HIV-1 infection, and only the two-exon form (Tat 86) can be phosphorylated by the preactivated double-stranded RNA-dependent kinase. Whereas the functional significance of this phosphorylation is still unclear, it has been suggested that it could promote the release of the protein from the substrate-binding region of the preactivated double-stranded RNA-dependent kinase (21).

Structural proteins, CAp24 and MAp17, have been shown to be phosphorylated. Phosphorylation of MAp17 is well documented. Indeed, MAp17 is phosphorylated on serine (22, 23) and tyrosine residues (24). Controversial results have been published concerning the function of those phosphorylations. It has been shown that the phosphorylation of serine residues of MAp17 would be essential to the association of the protein with the preintegration complex (25). Phosphorylation of the COOH-terminal Tyr residue (Tyr-131) of MAp17 would be implicated in the nuclear translocation of the preintegration complex and, thus, would be essential for the infection of non dividing cells (26, 27). However, participation of MAp17 during the infection of nondividing cells and the putative role of the phosphorylation of Tyr-131 in this process have been discussed by other authors (28–31). Although several studies have been published attempting to identify the cellular serine/threonine protein kinase implicated in MAp17 phosphorylation, results are still controversial. MAp17 has been identified as a substrate of protein kinase C (32), and this result is consistent with the identification of a highly conserved consensus protein kinase C phosphorylation motif in the HIV-1 Gag protein. Recently, the ERK/MAPK has been proposed to phosphorylate MAp17, al-
though there are no consensus ERK/MAPK recognition sites within MAP17 (33). Compared with phosphorylation of MAP17, phosphorylation of Cap24 has not been studied in detail. It has only been shown that Cap24 is phosphorylated on serine residues (22, 23). However, the phosphorylation sites of Cap24 are still unknown.

We previously reported the presence of two cellular serine/threonine protein kinases incorporated in HIV-1 particles (34). We have identified one of those protein kinases as MAPK ERK2, and this result has been recently confirmed by others (33). The identity of the other protein kinase is still unknown. We also showed that Cap24 is phosphorylated on serine residues by one of those protein kinases.

In this study, we showed that MAPK ERK2 does not phosphorylate Cap24. We demonstrated by site-directed mutagenesis that the three major phosphorylation sites of Cap24 were Ser-109, Ser-149, and Ser-178. Furthermore, by investigating the function of this phosphorylation during viral infection, we showed that this phosphorylation was not implicated in viral assembly, budding, or Gag protein maturation processes. By contrast, we demonstrated that phosphorylation of Cap24 on these residues was directly implicated at the early steps of infection, during the reverse transcription process. These results reveal a new regulation level of HIV-1 infection.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNAs and Site-directed Mutagenesis**—The parental viral DNA clone used in these studies is the biologically active plasmid pNL4-3 (35) and was propagated in Escherichia coli HB101 (clone 1035). The Unique Site Elimination mutagenesis system (Amersham Pharmacia Biotech) was used for in vitro mutagenesis of the Cap24 protein as described by the supplier. The 1.3-kilobase pair BssHII/ApaI fragment encoding for the Gag proteins was removed from pNL4-3 plasmid containing a complete infectious clone of HIV-1 and subcloned into the BssHII and ApaI sites of pET-11c (New England Biolabs, Inc.). The resulting construct pET-11c/gag was used for site-directed mutagenesis. Each of the 9 serine residues of Cap24 was substituted by an alanine residue using the target mutagenic primers listed in Table I. The mutant (SP) was subcloned back in pNL4-3 using Eco I. coli DH5α, pNL4-3, and its SP derivatives were propagated in HB101 (clone 1035) to prevent recombinations. Supercopied plasmids were purified with the Plasmid Mega kit (Qiagen).

**Phosphorylation Assay**—Equivalent amounts (1 µg) of GST, GST-p24 (kindly provided by V. Tanchou, Ecole Normale Supérieure, Lyon, France) and GST-EKf fusion proteins (kindly provided by D. A. Brenner, University of North Carolina, Chapel Hill, NC), produced in E. coli DH5α, were immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech). Beads were then incubated with 20 ng of activated MAPK ERK2 (Stratagene) for 2 h at 4 °C and washed five times in HBB buffer (20 mM Heps, pH 7.7, 50 mM NaCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% Triton X-100, and Complete™, a protease inhibitor mixture from Roche Molecular Biochemicals). Phosphorylation assay was performed for 20 min at 20 °C by resuspending the beads in kinase buffer (50 mM Heps, pH 7.5, 5 mM MgCl2 containing 2 µCi of [γ-32P]ATP. The reaction was stopped by washing five times in cold HBB buffer. Proteins were then separated by SDS-15% PAGE, transferred to PVDF membrane (Immobilon-P, Millipore), immunoblotted with a polyclonal anti-ERK2 antibody (Santa Cruz), and autoradiographed to visualize phosphorylated proteins, as described elsewhere (34).

**Cell Culture, Transfections, and Virion Production**—Human kidney 293 cells (ATCC CRL-1573) and HeLa P4 cells (36) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), and 100 units/ml penicillin, 100 µg/ml streptomycin, 2 µM glutamine (and 50 µg/ml Geneticin for HeLa P4 cells). Human CD4+ T lymphocyte cell lines (H9, SupT1, and C8166) were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of two seronegative individuals using a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech). For viral infection assay, a mix of PBMCs from the two donors was stimulated with 10 µg/ml of phytohemagglutinin (Life Technologies, Inc.) for 72 h and then maintained in RPMI 1640 as described above supplemented with 20 units/ml interleukin 2 (Roche Molecular Biochemicals).

Viral proteins were expressed transiently by polyethyleneimine transfected 293 cells (36). E. coli DH5α was used for the induction of cytopathogenic effects by infecting 5 × 10⁴ C8166 cells with 100 µl of virus (at 10⁵ cpm/ml RT activity) or 10⁶ SupT1 cells with 2 ml of virus (at 2.1 × 10⁸ cpm/ml RT activity). The appearance of cytopathic effects on C8166 cells and SupT1 cells was scored, respectively, 3 and 6 days later.

Two × 10⁶ PBMC and 10⁵ H9 cells were exposed to 200 µl of the 293 cell-derived viruses at RT activity of 5.1 × 10⁷ cpm/ml for 1 h at 37 °C, washed with Hanks’ balanced solution, and plated in 2 ml of fresh medium in 24-well and 6-well plates, respectively. Experiments were repeated in duplicate in PBMCs and in triplicates with H9 cells. Virus infectivity was monitored by measurement of virion-associated RT activity from cell culture supernatants every 4 days, starting from 5 days post-infection.

**Reverse Transcriptionase Assay and p24 Antigen Enzyme-linked Immunosorbent Assay**—Supernatants from transfected or infected cells were harvested and assayed for virion-associated RT activity and p24 antigen content. Cell culture supernatants were cleared of cells and cellular debris by centrifugation. For each sample, 100 µl of viral DNA into 293 cells (4 × 10⁵ cells). Supernatants from transfected cells were renewed 3 days after transfection and collected 24 h later (day 4). At the end of the experiments, the samples were analyzed for reverse transcriptase (RT) activity, and 0.45-µm pore size-filtered viral stocks were stored at −80 °C until used. All experiments were done in triplicate.

The transfection efficiency was assessed 72 h after co-transfection of pCMV-CAT (Roche Molecular Biochemicals) and each proviral DNA using a CAT enzyme-linked immunosorbent assay (Roche Molecular Biochemicals).

For electron microscopy analysis, DNA transfections were performed on HeLa P4 cells by calcium phosphate precipitation method on cell suspension (37) using 10 µg of DNA for 3 × 10⁵ cells.

**Viral Infectivity Assays**—All viral stocks used in a given experiment were normalized for RT activity. Virus was tested for the induction of cytopathogenic effects by infecting 5 × 10⁴ C8166 cells with 100 µl of virus (at 10⁶ cpm/ml RT activity) or 10⁶ SupT1 cells with 2 ml of virus (at 2.1 × 10⁸ cpm/ml RT activity). The appearance of cytopathic effects on C8166 cells and SupT1 cells was scored, respectively, 3 and 6 days later.

Two × 10⁶ PBMC and 10⁵ H9 cells were exposed to 200 µl of the 293 cell-derived viruses at RT activity of 5.1 × 10⁷ cpm/ml for 1 h at 37 °C, washed with Hanks’ balanced solution, and plated in 2 ml of fresh medium in 24-well and 6-well plates, respectively. Experiments were repeated in duplicate in PBMCs and in triplicates with H9 cells. Virus infectivity was monitored by measurement of virion-associated RT activity from cell culture supernatants every 4 days, starting from 5 days post-infection.
a 50.2 Ti rotor (Beckman). Virions were dissolved in TEN (0.1 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA) and lysed with 2 × SDS gel-loading buffer (40). Virion-associated proteins, normalized for RT activity, were separated by electrophoresis on a 10% ProSieben50 polyacrylamide gel (FMC), then transferred to PVDF membrane (Immobilon-P, Millipore). Proteins were analyzed by immunoblotting using either anti-RT rabbit polyclonal antibodies (kindly provided by J. L. Darlix, Ecole Normale Supérieure, Lyon, France) or anti-CAp24 human monoclonal antibody (kindly provided by D. Bourrel, Establishment de Transfusion Sanguine, Lille, France) and an enhanced chemiluminescence detection kit (Pierce).

**Electron Microscopy Analysis**—Transfected HeLa P4 cells were processed for thin-layer electron microscopy as follows. Seventy-two hours post-transfection, cells were washed once with medium and fixed in situ with 2.5% glutaraldehyde in PBS for 30 min at 4 °C. Cells were then post-fixed with 1% osmium tetroxide and embedded in epon. Sections were counterstained with uranyl acetate and lead citrate and examined with a Philips CM 120 transmission electron microscope.

**In Vitro Phosphorylation Assay and Immunoprecipitation of CAp24**—293 cells were transfected with WT or mutant pNL4-3 plasmid. Forty-eight hours post-transfection, medium was renewed, and supernatants were harvested 24 h later. Sixteen ml of 0.45-μm pore size-filtered supernatant were centrifuged through a 4-ml cushion of 20% sucrose as described above. Viral pellets were dissolved in 50 μl of lysate buffer, and submitted to an in vitro phosphorylation assay as described elsewhere (34). Lysates were precleared by incubation in 500 μl of TLB buffer (1 μl Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 140 mM NaCl, 2 mM EDTA) containing 15 μl of protein A/G plus agarose (Santa Cruz) and 5 μl of heat-inactivated normal human serum for 1 h at 4 °C. After centrifugation for 5 min at 10,000 × g, supernatants were incubated for 1 h at 4 °C with 1 μg of anti-CAp24 human monoclonal antibody, and the CAp24 immunocomplexes were isolated after overnight incubation at 4 °C using 20 μl of protein A/G plus agarose, pelleted, and washed five times with 1 ml of TLB buffer. Twenty μl of 2 × SDS gel-loading buffer were added to pellet, boiled for 3 min, loaded, and separated on a SDS-15% PAGE. Phosphorylated proteins were transferred to PVDF membrane (Immobilon-P, Millipore), autoradiographed, and then immunodetected with an anti-CAp24 mouse monoclonal antibody (kindly provided by B. Verrier, Ecole Normale Supérieure, Lyon, France) revealed by chemiluminescence.

**Viral RNA Analysis**—Viral stocks from 293 transfected cells were concentrated through a 20% sucrose cushion. Pellets were resuspended in 200 μl of viral buffer (50 mM Tris, pH 7.4, 10 mM NaCl, 10 mM MgCl₂, and 6 mM CaCl₂), an aliquot was taken for measurement of RT activity, and the remainder was lysed in 1% SDS, 100 μg of proteinase K/ml, 10 units of RNase inhibitor (Promega) for 1 h at 37 °C. Nucleic acids were extracted twice with phenol-chloroform and once with chloroform, ethanol-precipitated, and resuspended in water. RNA from equivalent volumes of control and infected cells was extracted, reverse transcribed, and PCR amplified with a 5′-GGCCAGAGATGATGAC-A-3′ primer and a reverse primer corresponding to pUC18 sequences from pNL4-3 (41). Any samples containing contaminating plasmid DNA detectable by PCR amplification were discarded.

To normalize the quantity of total cellular DNA present in each sample, human GAPDH DNA was amplified under the conditions described above with a forward primer corresponding to nucleotides 70 to 93 (5′-GGCCAGAGATGATGAC-A-3′) and a reverse primer corresponding to nucleotides 430 to 406 (5′-GGCCAGAGATGATGAC-CCCTTGAG-3′).

**RESULTS**

The Cap24 Is Not a Substrate of ERK2 MAPK—We have previously showed that Cap24 is phosphorylated by one of the two cellular protein kinases incorporated in viral particles. Moreover, we have identified one of those serine/threonine kinases as the ERK2 MAPK. The other protein kinase (53 kDa apparent molecular mass) is still unidentified.

In order to determine whether Cap24 could interact with ERK2 MAPK and could be phosphorylated, a solid-state kinase assay was performed. Activated ERK2 MAPK was incubated with either GST-p24, GST, or GST-Elk fusion proteins immobilized on GSH-Sepharose beads in the presence of [γ-³²P]-ATP. As shown in Fig. 1, no direct interaction, and thus no phosphorylation, could be detected when activated ERK2 MAPK was incubated with GST-p24 or GST proteins. By contrast, GST-Elk, one of the known substrates of ERK2 MAPK, indeed interacted and was phosphorylated as expected.

These results demonstrate that Cap24 is not a direct substrate for ERK2 MAPK.

**Construction of Cap24 Substitution Mutants**—It has been shown that Cap24 is phosphorylated on serine residues. The Cap24 protein contains 9 highly conserved serine residues. In order to study which serine residues were phosphorylated, we generated by site-directed mutagenesis 9-mutant proviral clones in which each serine residue was substituted by an alanine residue (Fig. 2). The presence of mutated codon has been checked by sequencing each proviral clone.

**Effects of Cap24 Mutations on Particle Formation and Release**—In order to test the ability of the Cap24 mutants to...
assemble and release virions, 293 cells were transfected with WT or mutant proviral DNAs. At day 4 post-transfection, supernatants were harvested and analyzed for RT content. As shown in Fig. 3A, RT activities of mutant particles were comprised between 131 and 79% of WT particles. Regarding to the standard deviations, those values are not significantly different. Similar results were obtained by measuring p24 antigen content in cell culture supernatants (data not shown). Moreover, the transfection efficiency was similar as measured by CAT activity after co-transfection of each proviral DNA with pCMV-CAT (data not shown). Thus, production of all mutant viruses from the transfected 293 cells was not affected, suggesting that assembly and release were normal in those mutants.

It has been shown that the $p55^{gag}$ precursor is sufficient for budding and production of immature particles. In order to verify if the maturation process of $p55^{gag}$ has been completed in mutant particles, we performed a Western blot analysis of viral proteins contained in these particles. The maturation process of $p55^{gag}$ was detected with an anti-CAP24 antibody that also reacts with the $p55^{gag}$ and the intermediate processing product $p41^{gag}$. No significant difference between WT and mutant viruses was observed (Fig. 3B, lower panel). Similar amounts of CAP24 and no accumulation of Gag precursor were detected for each mutant compared with WT. To assess whether similar amounts of viral lysates were loaded, the blot was reprobed with an anti-RT polyclonal antibody (Fig. 3B, upper panel). Again, no significant difference was observed for the RT content of each viral particle. These results show that the maturation process has been properly achieved in all mutant viruses.

Thus, the CAP24 mutations did not appear to affect the late steps of the virus life cycle, such as protein processing, virus assembly, and release.

**State of Phosphorylation of Mutated CAP24**—In order to determine whether the mutations introduced in CAP24 have modified the state of phosphorylation of the protein, we performed a phosphorylation assay followed by immunoprecipitation of CAP24. Virus stocks produced from 293 transfected cells were lysed and incubated with [γ-32P]ATP. Phosphorylated CAP24 was then immunoprecipitated and visualized by autoradiography as described elsewhere (34). As shown in Fig. 3C, upper panel, all the capsid proteins were phosphorylated, with a significant decrease in signal for the SP11, SP14, and SP15 mutant viruses. Similar amounts of CAP24 were immunoprecipitated in each virus as shown in Fig. 3C, lower panel.

Thus, each mutation introduced at Ser-109, Ser-149, and Ser-178 reduced the phosphorylation level of CAP24.

**Infectivity of Virions Containing CAP24 Mutations**—To determine whether the mutant particles were infectious, H9 and PBMCs were incubated with WT or mutant virions that had been harvested from supernatants of 293 transfected cells. Culture medium was harvested every 4 days and tested for virion-associated RT activity.

As shown in Fig. 4A, viral replication peaked at day 16 for the WT, SP1, SP3, SP10, SP12, and SP13 viruses. A delay was observed with the SP2 mutant virus. By contrast, no replication could be detected when SP11, SP14, and SP15 mutant viruses were tested, even after 40 days of culture. Cytotoxic effect of all mutant viruses was also examined with C8166 or SupT1 cells, showing that SP1, SP14, and SP15 mutant viruses were unable to induce syncytia formation in these cells.
or PBMCs (were normalized for RT activity and used to infect the H9 T-cell line (tained by transfection of 293 cells with the indicated molecular clones
independent experiments with similar results.

supernatant over time. All data presented are representative of three

virion RNA was quantified. WT, SP11, SP14, and SP15 viruses

alanine substitution of Ser-109, Ser-149, or Ser-178 affects the

viruses has been studied by electronic microscopy. SP11, SP14,

whether the lack of infectivity of SP11, SP14, and SP15 mutant

viral ultrastructure or the RNA packaging. We then studied

infectious ability of virions without modifying significantly the

alanine substitution of Ser-109, Ser-149, or Ser-178 affects the

altered and probed with an α-32P-labeled fragment from the Gag

region. Known quantities of RNA from WT virus were used to

establish a standard curve and to determine RNA quantity

within mutant particles (Fig. 5A). Virion RNA incorporated in

SP11, SP14, and SP15 mutant viruses were at 47.9, 59.5, and

42% of the WT virus, respectively. Thus, mutant particles were

able to incorporate viral RNA with an efficiency slightly lower

than the WT virus. However, it is unlikely that such a reduc-

tion in RNA incorporation could generate a total lack of infec-

tivity as observed in H9, C8166, and SupT1 cell lines or

PBMCs.

PCR Analysis of Viral DNA Synthesis—We have shown that

alanine substitution of Ser-109, Ser-149, or Ser-178 affects the

infectious ability of virions without modifying significantly the

viral ultrastructure or the RNA packaging. We then studied

the steps of reverse transcription process by PCR after entry of

viral particles into target cells.

SP11, SP14, and SP15 mutant viruses were produced from

293 transfected cells, treated by DNase I to remove any plas-

mid contaminant, normalized for RT activity, and used to infect

SupT1 cells. DNA was extracted from SupT1 cells 2, 6, and 24 h

post-infection and used as a template in PCRs with primer

pairs designed to amplify the products of reverse transcription.

In order to check the absence of plasmid DNA contaminating

samples, all samples were tested with a primer pair that spe-

cifically amplify the pUC vector (41), and all samples found

positives were discarded.

The primer pair 5'-R7'-U5 annealed with the early interme-

diate products of reverse transcription: the minus- and the

plus-strand strong stop DNA; primer pair 5'-R/MA detected the

second-strand transfer and the elongated positive-strand DNA;

primers U3pos.1/3'-ASPBS enabled to amplify the com-

plete double-stranded viral DNA. Finally, the nuclear double

LTR circular form was detected by PCR with the primer pair

LTR-U5/LTR-R followed by a second PCR with the internal

primer pair N-LTR-U5/N-LTR-R.

As shown in Fig. 5B, the first two steps of reverse transcrip-
tion tested were detected for all three mutant viruses at 24 h post-infection. Same results were obtained at the 2- and 6-h post-infection points (data not shown). No signal detection for the full-length cDNA could be observed when SP14 and SP15 viruses were used to infect SupT1 cells. By contrast, this DNA intermediate could be amplified after infection of cells with the SP11 mutant or the WT viruses. The next reverse transcription step is characterized by the translocation of the double-stranded cDNA from the cytoplasm to the nucleus. As expected, no signal was observed for SP14 and SP15 mutant viruses, whereas no nested PCR product could be detected with SP11 mutant virus.

These results show that substitution of Ser-109, Ser-149, and Ser-178 blocks the infection process during the early steps of the viral life cycle at the reverse transcription level.

**DISCUSSION**

In this report, we have shown that CAp24 is not a direct substrate of the virion-associated cellular protein kinase ERK2 MAPK and that Ser-109, Ser-149, and Ser-178 are essential amino acids required during the early steps of cellular infection.

It has been shown that CAp24 could be phosphorylated by a virion-associated protein kinase. Two cellular protein kinases are incorporated in viral particles: the ERK2 MAPK and a 53-kDa protein kinase (34). In this study, we have demonstrated that ERK2 MAPK cannot phosphorylate directly CAp24. This result is consistent with the one from Jacqué et al. (33) showing that activators and inhibitors of the ERK2 MAPK pathway have no effect on the phosphorylation of CAp24. Taken together, these findings suggest that CAp24 could be phosphorylated by the 53-kDa protein kinase. Identification of this protein kinase could allow us to confirm this hypothesis. Further experiments need to be done to isolate and sequence this protein before any assessment could be achieved on its role during the phosphorylation process of the viral protein.

We then focused our study on the identification of the phosphorylation sites of CAp24 and their putative role in the HIV infection cycle. Generation of mutant viruses allowed us to emphasize the essential function of three serine residues: Ser-109, Ser-149, and Ser-178. Substitution of one of these serine residues significantly reduces the phosphorylation of the mutated CAp24. Difference in the state of phosphorylation of the WT and the mutated CAp24 would be due to the substitution of one serine residue, suggesting that Ser-109, Ser-149, and Ser-178 were phosphorylated in the WT CAp24. This is the first evidence concerning the identification of the phosphorylation sites of CAp24. Indeed, phosphorylation of CAp24 has been demonstrated 10 years ago (22, 23), and since then, nothing new has been published on this topic. The fact that the phosphorylation of mutated CAp24 is only reduced implicates that the two other nonsubstituted serine residues are still phosphorylated. Thus, it is likely that the conformation of the mutated CAp24 was not significantly modified by the substitution since, for each mutant virus SP11, SP14, and SP15, the two other phosphorylation sites are still reachable to the protein kinase.

Our results allowed us to clarify the role of CAp24 phosphorylation during the viral life cycle. Indeed, whereas the three mutant viruses SP11, SP14, and SP15 were able to assemble, bud, and present a cone-shaped capsid, they were unable to infect T cells. In fact, these viruses were able to enter the cells as shown by the presence of the first intermediate products of reverse transcription (Fig. 5B), but the reverse transcription process cannot be completely achieved. The difference in infectivity observed between SP11 and the two other mutants SP14 and SP15 is apparently cell-dependent. Indeed, the ability of SP11 mutant virus to infect cells depends on the target cells used (Fig. 4). This suggests that a cellular function is able to make up for the phosphorylation of Ser-178. Furthermore, PCR analysis of different reverse transcription steps showed a difference between SP11 and SP14 or SP15 viruses. In the case of SP11 mutant virus, the reverse transcription process is able to progress till the synthesis of the full-length cDNA, whereas SP14 and SP15 mutant viruses were not able to perform reverse transcription till this point (Fig. 5B). This could be explained by the fact that phosphorylation of Ser-109 and Ser-149 (SP14 and SP15, respectively) was required before phosphorylation of Ser-178 (SP11). Phosphorylation of those 3 residues might occur during the reverse transcription process. Without the phosphorylation of Ser-109 and Ser-149, reverse transcription could not progress after the second strand transfer, whereas phosphorylation of Ser-178 would be necessary after the synthesis of the full-length cDNA and thus before the translocation of the viral DNA into the nucleus. Taken together, these results suggest that phosphorylation of CAp24 takes place at a very early step of the cellular infection.

Several reports have used mutagenesis to study the functional role of the different domains of CAp24, but none have modified the Ser-109, Ser-149, or Ser-178 residues. Regarding the localization of these three serine residues, none is implicated in a secondary structure such as a helix or β hairpin (42, 43). It is admitted that CAp24 is constituted by two domains: an amino-terminal domain (1–146 residues), whose refolding is implicated in the formation of the cone-shaped core (44, 45), and a carboxyl-terminal domain (151 to 231 residues), which is implicated in the dimerization of the CAp24. The several amino acids located between this two domains (146–151 residues) are necessary for forming the high-affinity capsid dimer interface (43). Modification of one of those two functions leads to a structural default of the viral particles as shown by studies using mutagenesis. SP11, SP14, and SP15 viruses do not present any structural defaults. Furthermore, Ser-109 is located in the amino-terminal domain of the CAp24 and is likely to be exposed to the surface of the capsid (44), Ser-149 is located in the linker previously cited, and Ser-178 is located in the carboxyl-terminal domain, on the inner side of the cone-shaped structure (45). Surprisingly, although those 3 serine residues were located in different functional domains of the CAp24, their substitution led to similar phenotypes regarding to the late steps of viral life cycle and the morphogenesis of the virion. This observation suggests that phosphorylation of these residues is necessary for a mechanism different from the ones already described.

Many studies have demonstrated the phosphorylation of viral proteins, but little is known about the phosphorylation of structural proteins. For example, the core protein of hepatitis B virus, that constitutes the capsid of the virus, is phosphorylated on serine residues (46, 47), and although the phosphorylated residues have been identified and several protein kinases have been proposed as candidate for performing this phosphorylation (48–51), the function of this phosphorylation is still unclear. In the case of the HIV capsid, we have shown that phosphorylation of CAp24 is necessary just after the entry of the virus in the target cell. At this point, two processes occur: the reverse transcription of viral RNA and the translocation of the viral DNA into the nucleus. We have demonstrated that with the phosphorylation mutant viruses, the reverse transcription process could not be completely achieved. This suggests that the phosphorylation could be implicated in the disassembly of the viral capsid. The detection of the first intermediate reverse transcripts is consistent with the fact that the reverse transcription has been shown to take place prior to or during the formation of the mature viral particle (52, 53).
Thus, the reverse transcription process does not depend on the dissociation of the core structure, and phosphorylation of Ser-109, Ser-149, and Ser-178 could be necessary to release the reverse transcripts before their translocation to the nucleus. The addition of negatively charged phosphate group in a structure such as the viral capsid could generate some repulsion powers and destabilize the capsid. Implication of phosphorylation in such a case has already been demonstrated for the tegument proteins of herpes simplex virus. Morrison et al. (54) showed that phosphorylation of structural proteins can promote the dissociation of the viral tegument. If the phosphorylation of CAp24 by a virion-associated kinase is necessary for the disassembly of viral core, then there is certainly a third component to the system. Indeed, the presence of the two components in a mature viral particle should destabilize the structure before the infection. Nevertheless, we showed that CAp24 could be phosphorylated by a virion-associated kinase inside the virion. This could be due to a low level of activation of this kinase, and when the viral particle attaches to the target cell, a stimulus would be created to completely activate the kinase and thus trigger the total disassembly of the viral capsid. This could allow the phosphorylation process to start at the proper time. For example, it has been shown that the interaction between gp120 and CD4 is able to activate cellular kinases implicated in signal transduction (55–59). One of these kinases could be the stimulus useful to trigger the phosphorylation of CAp24 and thus the disassembly of viral core.

The assembly process has been well documented for the past few years (60), whereas little is known about the uncoating process and the very early events of cellular infection. It has been proposed that cyclophilin A could play a role in dissociation of CAp24 (61, 62). It could be interesting to study the putative interaction between cyclophilin A and the phosphorylated CAp24. Does cyclophilin A interact with the cellular protein kinase that phosphorylates CAp24? Nothing is known about the putative phosphorylation of cyclophilin A. The phosphorylation status of the CAp24 and/or the cyclophilin A could modify the stability of the core structure. Answering these questions could clarify the first steps of the cellular infection and suggest a possible level of regulation to block the infection.

Acknowledgments—We thank J.-L. Darlix, V. Tanchou, and Y. Goldberg for helpful discussions. We thank S. Peyrol and I. Racine for technical assistance for electron microscopy analysis.

REFERENCES

1. Henderson, L. E., Bowers, M. A., Sewder, R. C. II, Serahy, S. A., Johnson, D. G., Bess, J. W., Arthur, L. O., Bryant, D. K., and Fenselau, C. (1992) J. Virol. 66, 1856–1865
2. Hunter, E. (1994) Semin. Virol. 5, 71–83
3. Spearman, P., and Ratner, L. (1996) J. Virol. 70, 8187–8194
4. Reicin, A. S., Ohagne, A., Yin, L., Hoglund, S., and Goff, S. P. (1996) J. Virol. 70, 8645–8652
5. Bokris, R. K., and Goff, S. P. (1996) Mol. Biol. 30, 303–305
6. Friborg, J. (1997) J. Biol. Chem. 272, 19441–19450
7. Li, D., and Darlix, J.-L. (1998) J. Virol. 72, 5867–5874
8. Fan, D. F., and Darlix, J.-L. (1998) EMBO J. 17, 7467–7474
9. Morrison, E. E., Wang, Y. F., Wang, C. S., and Meredith, D. M. (1998) J. Biol. Chem. 273, 10121–10129
10. Biagi, L., Signoret, N., Gaubin, M., Robert-Hebmann, V., Zhang, X., Murali, R., Greene, M. I., Piatier-Tonneau, D., and Devaux, C. (1997) J. Virol. 71, 8477–8484
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Kinch, K., and Ting, L. P. (1998) J. Virol. 72, 5067–5074
13. Coates, K., Cooke, S. J., Mann, D. A., and Harris, M. P. G. (1997) J. Biol. Chem. 272, 12328–12334
14. May, R., Bouny, V., Malim, M. H., and Cullen, B. R. (1988) J. Virol. 62, 4891–4894
15. Cecchini, A. W., Kramer, R., Ruben, S., Levine, J., and Rosen, C. A. (1988) Virology 171, 264–266
16. Meggio, F., D’Agostino, D. M., Cinimale, V., Chieco-Bianchi, L., and Pianna, L. A. (1996) Biochem. Biophys. Res. Commun. 226, 547–553
17. Fouts, D. E., True, H. L., Cengel, K. A., and Celander, D. W. (1997) J. Virol. 71, 2323–2328
18. Veronese, F. M., Copeland, T. D., Oroszlan, S., Gallo, R. C., and Sarnagharna, M. G. (1988) J. Virol. 62, 285–296
19. Mervis, R. J., Ahmad, N., Lillehoj, E. P., Raahemifar, H. F., Chan, H. W., and Venkatesan, S. (1988) J. Virol. 62, 3993–4002