Active cAMP-dependent Protein Kinase Incorporated within Highly Purified HIV-1 Particles Is Required for Viral Infectivity and Interacts with Viral Capsid Protein*

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Host cell components, including protein kinases such as ERK-2/mitogen-activated protein kinase, incorporated within human immunodeficiency virus type 1 (HIV-1) virions play a pivotal role in the ability of HIV to infect and replicate in permissive cells. The present work provides evidence that the catalytic subunit of cAMP-dependent protein kinase (C-PKA) is packaged within HIV-1 virions as demonstrated using purified subtilisin-digested viral particles. Virus-associated C-PKA was shown to be enzymatically active and able to phosphorylate synthetic substrate in vitro. Suppression of virion-associated C-PKA activity by specific synthetic inhibitor had no apparent effect on viral precursor maturation and virus assembly. However, virus-associated C-PKA activity was demonstrated to regulate HIV-1 infectivity as assessed by single round infection assays performed by using viruses produced from cells expressing an inactive form of C-PKA. In addition, virus-associated C-PKA was found to co-precipitate with and to phosphorylate the CAp24 MAPK protein. Altogether our results indicate that virus-associated C-PKA regulates HIV-1 infectivity, possibly by catalyzing phosphorylation of the viral CAp24 MAPK protein.

Protein phosphorylation is one of the primary processes by which external physiological stimuli influence intracellular events in eucaryotic cells. Protein kinase activity was also reported to contribute to a cascade of events controlling the definition of infectivity for a number of retroviruses and non-retroviruses. Many purified virions, including insect viruses, as granulosis virus (1), and plant viruses, including cauliflower mosaic virus (2), have been found to display protein kinase activity associated with their viral particles. For animal viruses, association of protein kinase activity with viral particles has been frequently reported, especially for enveloped virus particles (for review see Ref. 3). Among these, some virion-associated protein kinases have been identified as virally encoded products. As an example, herpes simplex viruses encode their own ribonucleotide reductases (ICP6 for HSV-1 and ICP10 for HSV-2), which have been assigned a serine-threonine protein kinase activity (4, 5), and similar observations have been performed for the human cytomegalovirus (6), pseudorabies virus (7), and varicella-zoster virus (8). For some small genome viruses, encapsidation of cellular kinases was found to replace their lack of kinase gene. Indeed, Rhabdoviridae (9), Hepadnaviridae (10), Retroviridae including Rauscher murine leukemia virus (11), and RNA tumor viruses (12) were found to incorporate host cell protein kinases within their membrane or inside the viral core. Even though their precise contribution has not yet been identified, there is ample evidence to suggest that virus-associated protein kinases are crucial for viral infectivity, and their possible function includes both the regulation of the viral nucleic acid replication and transcription and the modification of virus structural proteins that leads to either uncoating or encapsidation of viral nucleic acids.

For the human immunodeficiency virus type 1 (HIV-1),1 the incorporation of signaling molecules including cellular protein kinases within viral particles was also questioned. HIV-1 virions were found to contain several phosphoproteins including MAP17 p65 (13), CAp24 MAPK (14), and recently p6 MAPK proteins (15). The viral matrix MAP17 p65 is phosphorylated by virus-associated kinase(s) at various steps of the HIV-1 replicative cycle (16). C-terminal phosphorylation of a tyrosine residue within MAP17 p65 protein during and immediately after virus production was proposed to facilitate the dissociation of viral matrix protein from the membrane phospholipids of the nascent virus particle prior to and during virus assembly (17, 18). Additional phosphorylation on serine residues was next reported and was proposed to promote membrane dissociation of the reverse transcription complex from the cell membrane at the site of entry, allowing its nuclear translocation (16, 19). Such modifications were found to occur in preintegration complexes isolated from target cells and in native virions, suggesting that host cell serine-threonine kinases might possibly be incorporated within HIV particles (16).

CAp24 MAPK protein was also proposed to be phosphorylated by a virus-associated serine-threonine kinase. Such phosphorylations of CAp24 MAPK protein on serine residues were reported to be required for viral infectivity, because the reverse transcription process is unable to complete in CAp24 MAPK mutants with

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; PKA, cAMP-dependent protein kinase; C-PKA, catalytic subunit of PKA; ERK, extracellular signal-regulated kinase; PVDF, polyvinylidene difluoride; mAb, monoclonal antibody; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide.
PKA Activity Associated with HIV-1 Particles

The concentration of proteins in viral lysates was normalized by quantification of CAp24\(^{\text{gag}}\) antigen concentration, with anti-CAp24\(^{\text{gag}}\) enzyme-linked immunosorbent assay (Beckman Coulter).

PKA in Vitro Kinase Assays—PKA activity in viral and cellular lysates was assayed using a PKA assay kit (Upstate Biotechnology, Inc.). Briefly, the lysates were incubated for 30 min at 30 °C with 100 μM Kempide, a PKA-specific substrate, and 10 μCi of \(\gamma^{\text{32P}}\)ATP in kinase buffer provided by the kit manufacturer, supplemented or not with PKA inhibitor peptide. The phosphorylated substrate is separated from the residual \(\gamma^{\text{32P}}\)ATP using P81 phosphocellulose paper and quantitated by using a liquid scintillation counter.

Western Blot Analysis—The cells were washed twice in cold phosphate-buffered saline and lysed in 60 μL Tris-HCl, pH 8, 1% Triton X-100, 1% sodium deoxycholate, 0.05% glutaraldehyde, 4% formaldehyde in 0.1 x Sörensen buffer, pH 7.4 (24), for 1 h at 4 °C. After washing, the cells were included in Lowry cryoM at −20 °C, sectioned, and processed for conventional electron microscopy or immunoelectron microscopy. Mouse monoclonal anti-C-PKA antibody (Santa Cruz Biotechnology) was used as the primary antibody. Secondary antibodies conjugated to horseradish peroxidase were revealed by enhanced chemiluminescent detection kit (Super Signal; Pierce).

Reverse Transcriptase Assay and CAp24\(^{\text{gag}}\) Antigen-Linked Immunosorbent Assay—Supernatants of transfected or infected cells were harvested and cleared of cells and cellular debris by centrifugation. For each sample, 1 ml of culture supernatant was tested for RT activity using a synthetic template primer that permitted the RT to neosynthesize radioactive DNA, as previously described (23). The CAp24\(^{\text{gag}}\) antigen concentration was determined by enzyme-linked immunosorbent assay.

Electron Microscopy and Immunoelectron Microscopy—Thin layer electron microscopy was processed as follows. The cells were washed with medium and fixed with 0.05% glutaraldehyde, 4% formaldehyde in 0.1 x Sörensen buffer, pH 7.4 (24), for 1 h at 4 °C. After washing, the cells were included in Lowry cryoM at −20 °C, sectioned, and processed for conventional electron microscopy or immunoelectron microscopy. Mouse monoclonal anti-C-PKA antibody (Santa Cruz Biotechnology) and M35/2F8 rat anti-p6\(^{\text{gag}}\) mAb (obtained from M. G. Sarnagardharr) (25) were used for immunoelectron microscopy, with the corresponding colloidal gold-labeled complementary antibodies, 10-nm gold-tagged anti-mouse Ig antibody, and 5-nm gold-tagged anti-rat, respectively.

For conventional electron microscopy, the cells were fixed with 2.5% glutaraldehyde in 0.1 x phosphate buffer, pH 7.5, postfixed with osmium tetroxide (2% in H\(_2\)O\(_2\)) and treated with tannic acid (0.5% in H\(_2\)O). After dehydration, the specimen were embedded in Epon (Epok 812, Fullum), and sections were stained with 2.6% alkaline lead citrate and 0.5% uranyl acetate in 50% ethanol. The sections were observed under an Hitachi HU7100 electron microscope.

Viral Infectivity Assays—MAGI Cells (21) that stably express the β-galactosidase reporter gene cloned downstream of the HIV-1 long terminal repeat promoter were plated at 8 x 10\(^3\) cells/ml in 24-well plates. The cells were exposed to HIV stock solutions normalized according to RT activity. 48 h post-infection, virus infectivity was monitored by measurement of β-galactosidase activity from the cell lysates as previously described (28). Briefly, 200 μL of total cellular extracts were measured for 4.5 h at 37 °C and 100 μL of 100 mM Na\(_2\)HPO\(_4\), 10 mM MgCl\(_2\), 1 mM 2-mercaptoethanol, and 6 mM o-nitrophenyl β-D-galactopyranoside. The β-galactosidase activity was evaluated by measuring absorbance at 410 nm and was normalized according to total protein content in the cell lysate. Viability of cells exposed to viruses produced in the presence of H89 was assessed as previously described (27). Briefly, the cells were incubated in buffer containing 1.5 x 10\(^5\) mAbs (ICN) and 50 μl of protein G-conjugated magnetic beads (Miltenyi Biotech). After 2 h at 4 °C, immune complexes were separated on magnetic columns. For in vitro kinase experiments, immunoprecipitates were incubated in the presence of 2.5 μg of recombinant CA-PKA, 5

The concentration of proteins in viral lysates was normalized by quantification of CAp24\(^{\text{gag}}\) antigen concentration, with anti-CAp24\(^{\text{gag}}\) enzyme-linked immunosorbent assay (Beckman Coulter).
we found that p56
preparation of purified viruses (data not shown). In contrast,
specific for PKR, ERK-1, GSK3, PI3K, CK2, CSK, and c-Src
a number of antibodies directed to cellular kinases. Antibodies
previously described (28). Viral lysates were then probed with
trypsin and isolated by sucrose density ultracentrifugation as
HIV-1 virions prepared from the supernatant of H9 cells in-
purified viruses were probed with antibodies specific for vari-

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We and others have previously reported that several ser-
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As shown in Fig. 1, concentration of virus-associated C-PKA by immunoblot-
ning. Concentrated supernatants of uninfected or HIV-1NL4-3-infected H9
cells digested by subtilisin or not and normalized according to
CAPaps levels were analyzed for C-PKA (A), ERK-2 (B), gp41env (C),
and CAPaps (D) content in immunoblotting experiments.

μCi of [γ-32P]ATP in the appropriate buffer containing 13.5 mM Mg2+,
90 μM ATP, and 2 μM cAMP for 30 min at room temperature. After several washes, phosphorylated products were eluted, loaded onto 15%
SDS-PAGE, transferred onto PVDF membrane, and revealed by autoradiography. Phosphorylated CAPaps was identified by incubating
the membrane with anti-CAPaps goat polyclonal serum (Biogenesis).

RESULTS

cAMP-dependent Protein Kinase Catalytic Subunit Is Specifically Incorporated within Highly Purified HIV-1 Viral Particles—We and others have previously reported that several ser-
ine-threonine and tyrosine kinases are packaged within HIV-1
particles. In an attempt to identify virus-associated protein kinase(s) incorporated within HIV-1 particles, lysates of highly purified viruses were probed with antibodies specific for vari-
ous cellular protein kinases in Western blotting experiments.
HIV-1 virions prepared from the supernatant of H9 cells in-
fected by NL4.3 virus strain were digested with subtilisin pro-
tease and isolated by sucrose density ultracentrifugation as
previously described (28). Viral lysates were then probed with
a number of antibodies directed to cellular kinases. Antibodies
specific for PKR, ERK-1, GSK3, P13K, CK2, CSK, and c-Src
protein kinases failed to detect the corresponding kinase in
preparation of purified viruses (data not shown). In contrast,
we found that p56lck tyrosine kinase is specifically associated
with subtilisin-digested HIV-1 particles (data not shown) as
previously observed by high pressure liquid chromatography
and Western blotting analysis (29). In addition, the presence of
a protein with a molecular weight of 40,000 was revealed by the
mean of a serum specific for the catalytic subunit of cAMP-dep-
endent protein kinase (C-PKA) (Fig. 1A, lane 4), suggesting
that C-PKA is incorporated within purified HIV-1 particles. As
previously reported, the main difficulties for characterization of
host cell proteins associated to viral particles lies in the
co-sedimentation of cellular microvesicles with viral prepara-
tions. For this particular reason, the specificity of our observa-
tion was ascertained by analyzing the presence of C-PKA sub-
unit in mock virus preparations (culture supernatants from H9
infected cells) that were prepared in the same manner as
virus lysates (Fig. 1A, lane 3) without any detectable staining.
Our results indicate that the presence of contaminant C-PKA
proteins evidenced from undigested preparations of mock virus
supernatants (visualized from Fig. 1A, lane 1), because of the
co-sedimentation of microvesicles with HIV-1 viral particles, is
completely removed by digestion of purified virions with pro-
tease. To ascertain the relevance of our results, the immunoblot
was then reprobed with anti-ERK-2 antibody (Fig. 1B). As
previously reported, the presence of the mitogen-activated pro-
tein kinase ERK-2 was evidenced from purified virions (20) but
not from subtilisin-digested preparations of H9 supernatants.

The efficiency of subtilisin treatment was also ascertained by
analyzing gp41env envelope glycoprotein pattern in subtilisin-
digested viral preparations. As shown from Fig. 1C, a reduction
in the molecular masses of the proteins detected by anti-
gp41env serum was observed after subtilisin treatment, indicat-
ing that proteins outside the viral envelope have been effi-
ciently digested. It is worthwhile to notice that the size of the
C-PKA is not altered after digestion of viral preparations with
subtilisin, indicating that in contrast to gp41env, the size of
which is lowered from 41 to 22 kDa after treatment, C-PKA is
protected from enzymatic digestion. This result suggests that
virus-associated C-PKA is located inside the virus particle.
Finally, the amount of protein loaded in each lane was con-
trolled by probing the membrane with anti-CAPaps mAbs (Fig. 1D).
Similar experiments performed from supernatants of cells chronically infected with HIV-2popt or SIV strains did not
allow the detection of the PKA catalytic subunit in the HIV-2
and SIV particles (data not shown). These results indicate that
the catalytic subunit of PKA is selectively incorporated within
HIV-1 particles.

The association of C-PKA to HIV-1 viral particles was then
controlled by immunoelectron microscopy after double labeling
of both chronically HIV-1-infected cells and free subtilisin-
digested viral particles. Chronically HIV-1-infected 8E5 cells,
that derive from a CEM parental cell line or CEM-uninfected
cells used as a control were embedded in Lowicryl, and the
sections were double labeled with anti-p60gag mAbs allowing
identification of viral structures and anti-C-PKA serum. Pri-
mary antibodies were revealed with anti-rat Ig or anti-rabbit Ig
secondary reagents coupled with 5- or 10-nm gold particles,
respectively. As shown in Fig. 2A, the sections obtained from
HIV-1-infected cells with anti-C-PKA mAbs revealed specific
structures labeled by anti-p60gag mAbs, and low background
was detected in the section of uninfected CEM cells (Fig. 2B).
These data confirmed the presence of PKA catalytic subunit
within HIV-1 virions. Similar observations were performed from
inclusions of cell-free virions previously digested with
subtilisin (data not shown). It is important to notice that label-
ing of the sections with anti-p60gag and anti-C-PKA allowed us
to monitor the efficiency of subtilisin digestion by comparing the
presence of microvesicles in untreated and subtilisin-
treated viral preparations.

cAMP-dependent Protein Kinase Incorporated within HIV-1 Viral Particles Is Catalytically Active—In the cell context, the
PKA activity is regulated by the association of C-PKA catalytic
subunits with two regulatory domains termed R-PKA that in-
hibit the catalytic properties of C-PKA. To further investigate
whether the C-PKA incorporated within highly purified HIV-1
particles is enzymatically active, the presence of R-PKA regu-

latory subunit was investigated by Western blotting exper-
iments performed from lysate of subtilisin-digested HIV-1 parti-
cles. In our experimental conditions, the presence of R-PKA
subunits could not be proved in preparations of viruses (data
not shown). In the absence of detection of virus-associated
regulatory R-PKA subunits, we next investigated for the
presence of virus-associated PKA enzymatic activity. Virus parti-
cles or subtilisin-treated virions were lysed and incubated in
appropriate buffer in the presence of Kemptide, a PKA syn-
thetic substrate. PKA kinase activity was measured by count-
ing the incorporation of [γ-32P]ATP in Kemptide. As shown in
Fig. 3A, phosphorylation of Kemptide was detected from lysate of
concentrated HIV-1NL4-3 particles and was maintained in

FIG.1. Detection of virus-associated C-PKA by immunoblot-
ing. Concentrated supernatants of uninfected or HIV-1NL4-3-infected H9
cells digested by subtilisin or not and normalized according to
CAPaps levels were analyzed for C-PKA (A), ERK-2 (B), gp41env (C),
and CAPaps (D) content in immunoblotting experiments.
subtilisin-digested viruses. Basal phosphorylation level measured in the absence of Kemptide is indicated for each sample. Specificity of PKA activity was next assessed by analyzing phosphorylation of Kemptide substrate in the presence of highly specific inhibitors of either PKA activity or protein kinase C/calmodulin kinase activity as a control. Incorporation of $[^{32}P]ATP$ within the substrate was abolished when PKA inhibitor peptide was added to the samples but not when protein kinase C/calmodulin kinase inhibitor mixture was added to the reaction mixture (Fig. 3B). Altogether, these data indicate that C-PKA kinase activity is associated with highly purified HIV-1 virions.

C-PKA Activity Affects HIV-1 Particles Production but Is Not Required for Viral Maturation or Assembly—We next studied whether virion-associated C-PKA activity affects viral infectivity. Virus particles were produced from cells cultured in the
removal (0, 2, and 4 h). The values correspond to one representative experiment of three. B, release of HIV-1 particles was quantified by measuring RT activity in culture supernatants from Cos-7 cells transfected with pNL4-3 and cultured in medium alone or in medium supplemented with 100 μM H89. H89 inhibitor was added to the cells for a 4-h time period 40 h post-transfection. The inhibitor was then removed, and the cells were maintained in culture for an additional 4 h. The supernatants were then assayed for RT activity (each value corresponds to the mean of six separate experiments).

**Materials and Methods**

The consequences of H89 treatment on viral product maturation were next investigated by Western blot experiments. Cell-associated viral proteins or protein content of samples prepared from supernatants of mock transfected cells or Cos-7 cells transfected with pNL4-3 and cultured in medium alone or medium supplemented with H89 were subjected to 10% SDS-PAGE and immunoblot analysis. The amounts of viral protein lysates were normalized for RT activity content. The blots were sequentially probed with rabbit anti-RT and anti-gp41 sera and anti-Cap24 mAbs, and the protein bands were visualized using chemiluminescent detection. The positions of migration of RT heterodimer (p51 and p69), Gag precursor (p55), intermediate processing product (p41), cleaved capsid protein (Cap24), and gp41 are indicated on the right.

**Fig. 4. Inhibition of C-PKA activity in host cell lowers HIV particle production.** A, analysis of PKA activity in Cos-7 cells treated or not with a PKA specific inhibitor (H89). The cells were treated for 4 h with 100 μM of H89, inhibitor was removed, and cellular PKA activity was measured as described under “Materials and Methods” from the cell lysates at different times after treatment removal (0, 2, and 4 h). The values correspond to one representative experiment of three. B, release of HIV-1 particles was quantified by measuring RT activity in culture supernatants from Cos-7 cells transfected with pNL4-3 and cultured in medium alone or in medium supplemented with 100 μM H89. H89 inhibitor was added to the cells for a 4-h time period 40 h post-transfection. The inhibitor was then removed, and the cells were maintained in culture for an additional 4 h. The supernatants were then assayed for RT activity (each value corresponds to the mean of six separate experiments).

**Fig. 5. Inhibition of host cell C-PKA activity do not interfere with expression and maturation of viral proteins.** Cell-associated viral proteins or protein content of viral particles produced from Cos-7 cells transfected with pNL4-3 and cultured in medium alone or in medium supplemented with 100 μM H89 were subjected to 10% SDS-PAGE and immunoblot analysis. Amounts of viral protein lysates were normalized for RT activity content. The blots were sequentially probed with rabbit anti-RT and anti-gp41 sera and anti-Cap24 mAbs, and the protein bands were visualized using chemiluminescent detection. The positions of migration of RT heterodimer (p51 and p69), Gag precursor (p55), intermediate processing product (p41), cleaved capsid protein (Cap24), and gp41 are indicated on the right.
PKA Activity Associated with HIV-1 Particles

Inhibition of virus-associated C-PKA activity affects HIV infectivity. Virus stocks obtained by transfection of Cos-7 cells with pNL4-3 vector cultured in medium alone or maintained in the presence of H89 were normalized for RT activity and were used at different concentrations to infect two different cell lines. A, infection of the MAGI indicator cell line was monitored by measuring 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) hydrolysis 48 h post-infection. Cell viability as determined by MTT testing 48 h after infection is shown in inset for each viral input used. B, virus replication in the H9 CD4⁺ T cell line was scored by measuring RT activity in cell supernatant. The upper panel represents the kinetic of replication of viruses (RT of viral input, 5000 cpm/ml) produced from pNL4-3-transfected Cos-7 cells maintained in medium alone (HIV + H9) or supplemented with 100 μM of H89 (HIV + H89). The lower panel represents the RT activities measured at day 10 after infection from the supernatant of H9 cells exposed to various concentrations of wild type or H89-treated viruses. Cell viability determined at day 10 post viral exposure by MTT cytotoxicity assay is shown as a control (inset). Each value is the mean of duplicate experiments.

virus-associated protein kinase has been previously shown to modulate virus infectivity (19). Implication of virion-associated C-PKA in HIV-1 infectivity was then assessed in a single cycle transactivation assay. Viruses collected from transfected Cos-7 cells maintained in the presence or in absence of H89 inhibitor were normalized for RT activity, and various virus concentrations were used to infect the MAGI indicator cell line. These cells expressing both HIV-1 receptor CD4 and co-receptors (CXCR4 and CCR5) and a β-galactosidase reporter gene driven by an HIV-1 viral long terminal repeat allowed us to quantify the efficiency of virus infection. After 2 days in culture, β-galactosidase activity was determined from the cell lysates. As shown in Fig. 6A, infection was barely detected from MAGI Cells exposed to viruses produced from H89-treated cells, whereas high X-galactosidase hydrolysis levels were observed from cells exposed to comparable levels of viruses produced in absence of PKA inhibitor. Differences in infectivity were observed at any given viral input tested (5000, 10,000, or 20,000 cpm of RT), with a marked difference when high infectious doses were used. To ascertain that differences in infectivity observed were not related to a cytotoxic effect of residual H89 present in the viral inoculum, cell viability was determined by MTT testing 48 h after exposure of the cells to the virus. As shown from Fig. 6, no significant cytotoxic effect was noted at any viral input used. This observation suggests that HIV-1 virions produced from cells with impaired C-PKA activity display a lower infectivity as compared with wild type viruses.

Infectivity of PKA-deficient virions was next analyzed by infection of H9 CD4⁺ T lymphoblastoid cells. The cells were exposed to various amounts of virions produced by transfection of Cos-7 maintained in the presence or absence of H89 inhibitor and normalized according to RT activity. After 1 h at 37 °C the cells were washed three times, resuspended in RPMI medium, and maintained in culture. The amount of virus produced by the cells was determined by measuring RT levels every 3 days from the cell supernatants. We observed that virus replication was significantly delayed when H9 cells were exposed to viruses produced from H89-treated cells, as compared with when these cells were exposed to wild type virus. Indeed, a delay of at least 3 days was observed for replication of viruses produced from C-PKA-deficient cells at any concentration tested (Fig. 6B, upper panel). As a representative example, values measured at day 10 after virus exposure for various concentrations of wild type and C-PKA-deficient viruses are shown in Fig. 6B (lower panel). The absence of cytotoxic effect of residual H89 contained in the viral inoculum was controlled at different times after virus exposure. As mentioned in Fig. 6B (inset), the differences observed between replication of wild type and C-PKA-deficient virions in H9 cells at day 10 after infection are not related to toxicity of the viral input as cell viability is unaffected. Despite this, after 14 days in culture, significant RT activities were detected from H9 cells exposed to H89-treated viruses. This result may reflect either the appearance or the propagation of replication efficient viruses. Such a phenomenon might be the consequence of an unstable inhibitory effect of H89 viral particles maintained in the culture medium. Consequently, the discrepancy observed between wild type and H89-treated viruses during follow-up of replication in H9 cells was significantly reduced at day 14 after viral exposure. Altogether our data indicate that viruses produced from cells expressing kinase-dead C-PKA, despite normal maturation and morphology, display reduced infectivity for CD4⁺ cells as compared...
with viruses produced from cells expressing fully active C-PKA.

The CAp24gag Protein Is a Substrate of C-PKA—Having demonstrated that active C-PKA associated with purified HIV-1 particles regulates viral infectivity, we attempted to explain its functional role. We have previously shown that several structural proteins of HIV-1 are phosphorylated by virus-associated kinases. Among these, the CAp24gag protein was found to be phosphorylated by a host cell serine-threonine kinase incorporated within viral particles (14). The contribution of ERK-2 viruse- associated kinase has been previously questioned, and its physiological relevance of this observation was estimated in an in vitro kinase experiment performed in the presence of nonrecombinant CAp24gag protein. CAp24gag was immunoprecipitated from H9 cells infected with the HIV-1NL4-3 strain and was used in an in vitro kinase experiment performed in the presence of recombinant C-PKA. We found that mature CAp24gag protein is phosphorylated in vitro by recombinant C-PKA (Fig. 7C). Altogether, these data support the hypothesis that CAp24gag is likely to be a substrate for C-PKA in vivo.

**DISCUSSION**

In the present study we demonstrated the selective incorporation of active catalytic C-PKA subunit within HIV-1 virions. Experimental approaches used to this end were based on protease digestion and sucrose density separation of virions. This method is efficient for removal of 95% of the microvesicle- associated proteins from virion preparations as assessed by inclusion of viral preparations and electron microscopy of thin layer sections. This strategy was previously used to report association to the inner face of HIV-1 virions of several host cell proteins including cytoskeletal components (28), glutaredoxin (30), and several actin-binding proteins (29). Our present result corroborates previous observations indicating that protein kinase activity is associated with HIV-1 particles as for a number of enveloped DNA- and RNA-containing animal viruses. The presence of two cellular serine-threonine protein kinases incorporated in HIV-1 parti-

**Fig. 7.** C-PKA interacts with and phosphorylates the CAp24gag protein. A, interaction of C-PKA and CAp24gag proteins was investigated by immunoprecipitation experiments. Immunoprecipitations (IP) were performed with lysates of uninfected or HIV-1NL4-3-infected H9 cells or with concentrated subtilisin-digested supernatants of these cells by using an anti-CAp24gag mAb or an irrelevant mAb (IR). After separation on 15% SDS-PAGE and transfer onto PVDF membrane, the membrane was successively probed with an anti-CAp24gag mAb (upper panel) and an anti-C-PKA serum (lower panel). The corresponding proteins are indicated by arrows. B, phosphorylation of CAp24gag by C-PKA was investigated in an in vitro kinase experiments by incubating 1 μg of recombinant CAp24gag protein with 100 μg of recombinant C-PKA and [γ-32P]ATP. The reaction mixture was supplemented or not with PKA inhibitor peptide (PKA Inh.). The reaction products were separated on 12% SDS-PAGE and transferred onto PVDF membrane, and phosphorylation of CAp24gag protein was revealed by autoradiography (32P). The membrane was successively probed with anti-CAp24gag mAbs and an anti-C-PKA serum to ascertain the protein content in each lane. The positions of phosphorylated CAp24gag (P-CAp24gag), CAp24gag, and C-PKA are indicated by arrows. C, CAp24gag protein was immunoprecipitated from lysates of HIV-1-infected H9 cells by using an anti-CAp24gag or an irrelevant mAbs (IR.). Immunoprecipitates were subjected to in vitro phosphorylation assay in the presence of recombinant C-PKA, separated on 15% SDS-PAGE, transferred onto PVDF membrane, and revealed by autoradiography (32P). Phosphorylated products corresponding to the CAp24gag protein (P-CAp24gag) were identified by probing the membrane with anti-CAp24gag mAbs and are indicated by arrows.
PKA Activity Associated with HIV-1 Particles

PKA activity associated to HIV-1 particles has previously been reported, and virus-associated ERK-2 was identified as being one of them (20). We describe here that C-PKA is an additional serine-threonine kinase associated to HIV-1 particles.

A very large spectrum of host cell components have been reported to be incorporated within HIV particles. For most of them, questions have been unanswered about their precise contribution to the viral life cycle, although they are known for their functional role in cells. Host cell proteins embedded in the viral envelope were mostly proposed to enhance virus affinity for its target cell. Functions for cytosolic virus-associated compounds are less clearly defined, and their packaging within viral particles helps to create an appropriate environment for propagating the virus into the target cell. The association with HIV-1 particles of C-PKA devoid of regulatory subunit and associated to enzymatic activity suggests a functional contribution for this protein during the retroviral life cycle. Indeed, virus-associated ERK-2 enzymatic activity was previously reported to play a predominant role in the establishment of a functional reverse transcription complex by phosphorylating MAPI17

**gag** protein, promoting dissociation of the reverse transcription complex and influencing its cellular localization in infected cells (19). We found here that impairment of host cell C-PKA activity by a synthetic inhibitor at the time of virus release results in the production of virions with reduced infectivity as assessed in single round infection assays performed in MAGI cells and in _in vitro_ infection of the CD4

**gag** lymphoblastoid H9 cell line. We cannot rule out the possibility that H89 inhibitor affects a late stage of virus replicative cycle, but the absence of modification of viral precursor maturation or virus assembly as shown by conventional biochemical and electron microscopy methods suggests that defects in viral infectivity might rely on the inhibition of virus-associated C-PKA activity. Virus-associated C-PKA might thus contribute to the regulation of HIV-1 infectivity.

It is also important to notice that C-PKA activity in the host cell was found to influence viral production. Indeed the addition of H89 inhibitor to cells expressing the pNL-3 plasmid was found to lower RT activities measured from the cell supernatants, suggesting a possible inhibitory effect on viral particle release. The role of cellular PKA is complex (for review see Ref. 31), and such alteration of viral production may be explained at different levels. First, PKA activity is required for regulation of host cell DNA-binding proteins involved in HIV transcription, including cAMP-responsive element-binding protein and NF-xB factors, which are affected by cAMP-dependent phosphorylation. Second, PKA activity was also found to participate in chromatin remodeling through histone phosphorylation. Therefore, PKA activity might interfere with both integration and transcription of HIV genome. Third, PKA regulates exocytosis, endocytosis, and transcytosis by acting on transport events from the endoplasmic reticulum to the plasma membrane across the Golgi stacks (32).

Because inhibition of PKA by the addition of H89 inhibitor to the cells was known to alter vesicle-mediated transport along the exocytic route, the experimental procedures used in the present study and aimed at producing virus having incorporated an inactive PKA were designed to limit the consequences of PKA inhibition on envelope glycoprotein release and incorporation into virions. To this end, H89 treatment of the cells was followed by the removal of H89 and a 4-h culture time period before recovery of the virus. Western blot analysis of cell-associated viral proteins and virions protein content from cells exposed or not to H89 demonstrated that similar levels of gp41

**gag** transmembrane proteins were incorporated in wild type and H89-treated virions, and viruses produced from cells cultured in the presence of efficient concentrations of PKA inhibitors were found to display fully mature protein content and morphologic characteristics similar to wild type virions. As a consequence we propose that the reduction of viral particle release observed from H89-treated cells is rather due to a direct consequence of inhibiting either transcription or integration steps of HIV-1.

Characterization of viral protein(s) targeted by virus-associated C-PKA will help to elucidate its precise contribution in HIV life cycle. C-PKA recognizes a consensus sequence (RX/ST/TX) that can be found in a number of HIV proteins including MapL17

**gag** , CAp24

**gag** , gp41

**gag** , Rev, Vpr, and Vif. In the present paper we report that the CAp24

**gag** protein is phosphorylated _in vitro_ by recombiant C-PKA, and the physiological relevance of this event was confirmed by co-immunoprecipitation of CAp24

**gag** and C-PKA in experiments performed from both cell extracts and virus lysates. The putative role of C-PKA in CAp24

**gag** phosphorylation is in agreement with our previous observations indicating that viral capsid protein is phosphorylated by virus-associated serine-threonine kinase distinct of ERK-2 (14).

Capsid phosphorylation is a common feature reported for a number of viruses and retroviruses. The herpes simplex virus type 1 tegument protein VP22 is phosphorylated on serine residues by both virion-associated and cellular kinases. Such phosphorylations were proposed as a regulatory mechanism in the dissociation of structural components of this virus (33, 34). Similar observations have been performed on the destabilizing effect that phosphorylations of the viral capsid have in uncoating of poliovirus (35). For HIV-1, CAp24

**gag** phosphorylations were found to participate in the early events of HIV replication, mainly in the late retrotranscription steps (14). Although the consequences of PKA-dependent phosphorylation of CAp24

**gag** remains to be defined, a possible implication of capsid protein phosphorylation in uncoating events can be proposed. Indeed, although evidence has been obtained from previous and present studies that CAp24

**gag** may be phosphorylated by a virus-associated kinase in _in vitro_ phosphorylation assays and that C-PKA may participate in such phosphorylation events, it remained difficult to prove the presence of phosphorylated forms of capsid protein in cell-free assembled HIV-1 mature particles either from one- or two-dimensional gel analysis (data not shown). This observation suggests that CAp24

**gag** phosphorylation by C-PKA may be a transient event, occurring at a precise stage of HIV life cycle. If CAp24

**gag** phosphorylation appeared to participate in the HIV-1 uncoating process, then the addition of negatively charged phosphate groups to capsid monomers could generate some repulsion force and destabilize the viral core. The contribution of PKA in such events is currently under investigation in our group.

In conclusion, host cell protein kinases associated to HIV-1 particles, including C-PKA, despite minor structural components of the viral architecture, might play an important role in the virus life cycle by modifying virion structural proteins, interfering with viral assembly, or uncoating and regulating the release of nucleic acid into the host cell. It is also important to consider that the presence of virus-associated C-PKA might be of special interest in deregulating the host cell activation level by triggering abnormal signaling. Interestingly, HIV infection was shown to up-regulate PKA activity and several CAMP-inducing mediators. In T cells from untreated HIV-infected patients, elevated levels of cAMP have been detected, and PKA subunits are constitutively activated (36). Identifying the precise relationship between the virus-associated protein kinases and the viral life cycle will reveal novel targets for the development of specific and new antiviral agents.
PKA Activity Associated with HIV-1 Particles

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