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Association of ERK2 Mitogen-Activated Protein Kinase with Human Immunodeficiency Virus Particles

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Here we report the presence of a protein kinase activity associated with human immunodeficiency virus type 1 (HIV-1) particles. We observed phosphorylation of five major proteins by the endogenous protein kinase activity. Phosphoamino acid analysis revealed phosphorylated serine and threonine residues. In addition, we observed autophosphorylation of two proteins in the presence of γ-ATP in an in-gel phosphorylation assay. These two proteins are not linked by a disulfide bond, suggesting that two different protein kinases are associated with HIV-1 virions. Our results indicate the presence of ERK2 mitogen-activated protein kinase and of a 53,000-molecular-weight protein kinase associated with virions. Moreover, the use of different HIV strains derived from T cells and promonocytic cells, as well as the use of human T-cell leukemia virus type 1 particles, demonstrates that ERK2 is strongly associated with retrovirus particles in a cell-independent manner. Exogenous substrates, such as histone proteins, and a viral substrate, such as Gag protein, are phosphorylated by virus-associated protein kinases.

Retroviruses rely on host factors for many aspects of their replication cycle, including transcription of the integrated provirus, translation of viral mRNAs, and posttranslational modifications of viral proteins. Several studies have shown that host cell proteins can be incorporated into budding human immunodeficiency virus type 1 (HIV-1) particles (2, 12, 21, 25, 33). Some of these virion-associated molecules may play a necessary functional role in resistance to immune effector mechanisms (2) or during cellular infection (12, 21, 33). The incorporated proteins include class I and II major histocompatibility complex and β2 microglobulin (2), cyclophilin A (12, 23), CD11a/CD18 (25), and CD44 (22). Host-derived receptors, such as CD11a/CD18, on virions could facilitate the adhesion of virus to target cells (25). Antibody to major histocompatibility complex class I and II molecules can also neutralize HIV (2). In addition, a correlation between virion-associated cyclophilin A levels and virion infectivity might exist at an early step of the HIV-1 replication cycle (3, 33). It is noteworthy that cyclophilin A is incorporated inside viral particles since it binds to the capsid (CA; $p24^{Gag}$) protein (23). Thus, host membrane molecules, as well as intracellular proteins, are associated with HIV particles.

Protein phosphorylation is one of the primary processes by which external physiological stimuli influence intracellular events in eukaryotic cells. Protein kinase activity has been demonstrated in a number of purified virions of enveloped DNA- and RNA-containing animal viruses. Many members of the herpesvirus group (28, 29) and retroviruses such as Rauscher murine leukemia virus (32) and RNA tumor viruses (17) have been shown to incorporate protein kinases in their membranes. However, other studies have suggested that protein kinases are associated with the cores of vaccinia virus (26) and hepatitis B virus (20). The function(s) of most virion-associated host protein kinases remains unknown; however, many are believed to be directly involved in viral replication. The possible functions of virus-associated protein kinases include regulation of viral nucleic acid replication and transcription, modification of virion structural proteins that leads to either uncoating or encapsidation of viral nucleic acid, and as-yet-undefined changes in host cellular proteins involved in viral replication. Virion-associated protein kinases might also trigger abnormal cell signaling, implicating dysfunctions of infected cells in response to different stimuli.

In the present study, a kinase assay was carried out with HIV virions. Virus was purified by multiple density gradients from HUT78 cells infected by $HIV\text{-}1_{HZ321}$ as already described (34). Viral lysate was obtained by solubilizing proteins in lysis buffer (50 mM HEPES [pH 7.5], 10 μM phenylarsine oxide, 200 μM sodium orthovanadate, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 5,000 U of aprotinin per ml, 0.5% Nonidet P-40) for 20 min on ice. The phosphorylation assay was performed with 50-μl reaction volumes containing 10 μg of viral lysate and 2 μCi of [γ-^{32}P]ATP in kinase buffer containing 50 mM HEPES (pH ranging from 5.0 to 8.0) and different concentrations of cations ($MnCl_2$, $MgCl_2$, and $CaCl_2$) supplemented or not with 100 mM EDTA or cyclic nucleotides as indicated (Table 1). The reaction mixture was incubated for 20 min at 20°C, stopped, precipitated by addition of 1 ml of 10% trichloroacetic acid–62.5 mM sodium pyrophosphate–2 drops of 0.5% human gamma globulin on ice, and centrifuged at 600 × *g* for 5 min. The precipitates were suspended in 100 μl of water, dissolved by the addition of 100 μl of NaOH, reprecipitated by the addition of 1 ml of 125 mM sodium pyrophosphate plus 1 ml of 20% trichloroacetic acid, washed with 5% trichloroacetic acid, and collected on Whatman GF/C filters. The samples were counted in a toluene-based scintillation cocktail in a Beckman liquid scintillation spectrometer. As shown in Table 1, protein kinase activity was dependent on the

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TABLE 1. In vitro characteristics of the virus-associated kinase activity

Dependence	Kinase activity (cpm)[a]
Cation	
EDTA, 100 mM	204 (3)
Mn^{2+}	
1 mM	6,495 (85)
5 mM	7,654 (100)
10 mM	6,469 (85)
Mg^{2+}	
1 mM	1,496 (20)
5 mM	2,644 (35)
10 mM	2,469 (33)
Ca^{2+}	
1 mM	2,418 (32)
5 mM	4,871 (64)
10 mM	2,496 (33)
pH	
5.0	4,246 (56)
6.0	4,582 (61)
7.5	7,654 (100)
8.0	6,843 (90)
Nucleotide	
None	10,129 (100)
cAMP	
5 μM	13,569 (134)
50 μM	10,491 (104)
500 μM	4,063 (40)
cGMP	
5 μM	8,672 (86)
50 μM	18,571 (183)
500 μM	33,514 (327)

[a] Parenthetical data are percentages of the control.

presence of the Mn^{2+} cation at from 1 to 10 mM. Magnesium chloride could not substitute for $MnCl_2$ efficiently, since 35% incorporation was the highest percentage obtained. The addition of Ca^{2+} ions did not increase the extent of phosphorylation. ^{32}P incorporation was inefficient when assayed at pH 5.0 or 6.0 but was active at pH 7.5 and still displayed activity at pH 8.0 (Table 1). The addition of cyclic AMP (cAMP) increased the level of ^{32}P incorporation at 5 μM and inhibited phosphorylation at 500 μM. The addition of cGMP stimulated the kinase protein at 50 and 500 μM by factors of 1.83 and 3.27, respectively (Table 1).

In order to visualize phosphorylated proteins, a kinetic analysis of phosphorylation was performed with the virus in the presence of γ-ATP and $MnCl_2$ (5 mM; pH 7.5) at 20°C. The phosphorylation assay was performed with 60-μl reaction volumes containing 50 μg of virus and 10 μCi of [γ-^{32}P]ATP in kinase buffer (50 mM HEPES [pH 7.5], 5 mM $MnCl_2$) at 20°C. The phosphorylation reaction was stopped at different times by adding 15 μl of 5× Laemmli sample buffer. Phosphorylated proteins were separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) and detected by autoradiography. Phosphorylation of four proteins with M_rs of 53,000, 43,000, 17,000, and 15,000 (53K, 43K, 17K, and 15K, respectively) could be detected as early as 1 min (Fig. 1A). After 5 min, another phosphorylated protein, 24K, was revealed. It should be noted that a strong signal was given by the 53K and 43K proteins from a 10-min incubation. Thus, the optimal conditions for the kinase assay are 20 min, 5 mM $MnCl_2$, and pH 7.5 in order to get the complete phosphorylation profile. Under these conditions, the addition of 500 μM cGMP did not increase the extent of phosphorylation of any protein (data not shown), showing that the observed 3.27-fold increase in ^{32}P incorporation (Table 1) did not correspond to a significant increase in protein phosphorylation. In addition, we did not detect any phosphorylated protein in the presence of γ-GTP as the phosphate donor (data not shown). Phosphoamino acid analysis was performed by standard procedures (9) and showed a preponderance of phosphorylated serine and small amounts of phosphorylated threonine (Fig. 1B). Thus, the virus-associated protein appears to be a serine/threonine protein kinase. The same profile of phosphorylation was de-

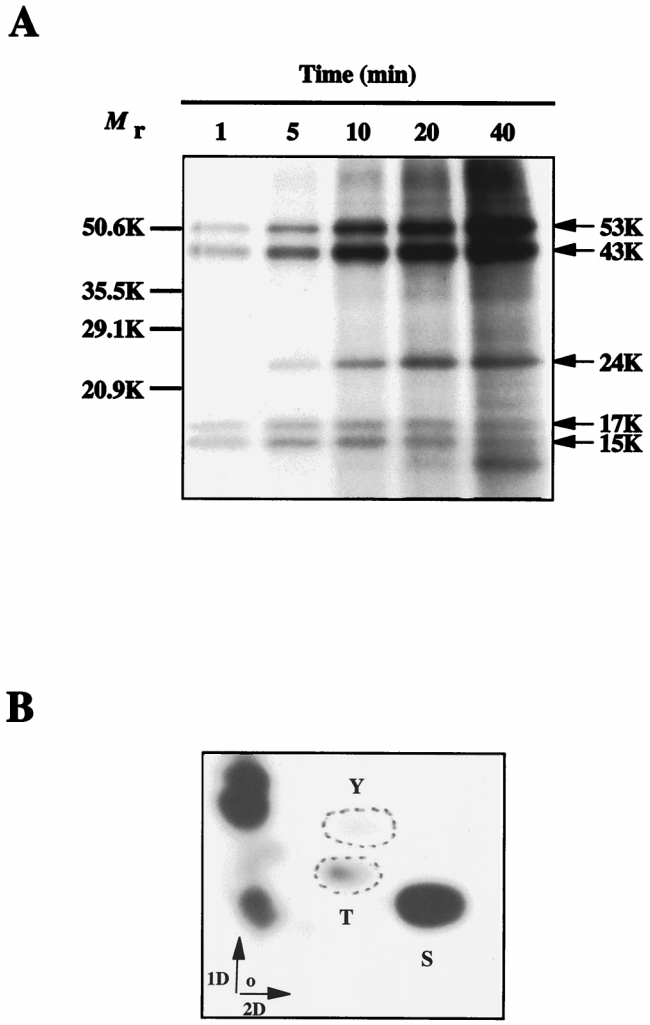

FIG. 1. Virion-associated protein kinase activity. (A) Autoradiograph after SDS–15% PAGE showing a kinetic study of phosphorylation of viral lysate proteins. The positions of relative molecular mass (M_r) markers are indicated on the left. (B) The phosphoamino acid content of the 43-kDa phosphorylated protein was determined in parallel experiments in which ^{32}P-labelled 43-kDa protein was excised from the polyvinylidene difluoride membrane prior to acid hydrolysis and two-dimensional (2D) chromatography. The positions of the phosphoamino acid standards (phosphoserine [S], phosphothreonine [T], and phosphotyrosine [Y]) and origin (○) are marked. The phosphoamino acid analysis results for all other bands were essentially identical to those shown.

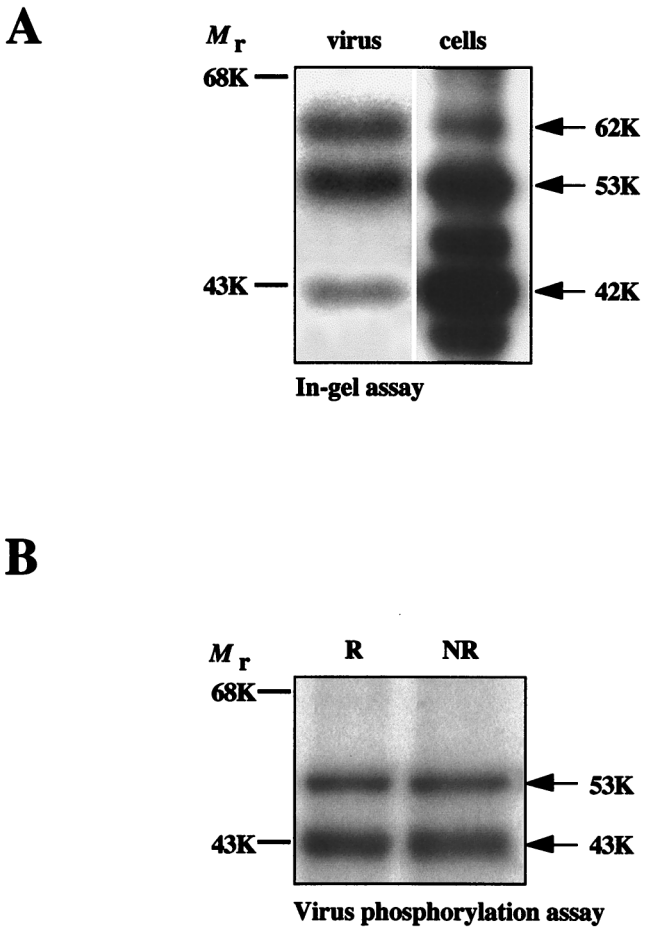

FIG. 2. Determination of the phosphorylated proteins showing kinase activity. (A) Autophosphorylation of protein kinases associated with virions, detected by an in-gel assay. (B) Phosphorylation assay of the viral lysate under reducing (R) and nonreducing (NR) conditions. The positions of relative molecular mass (M_r) markers are indicated on the left.

tected in two independent preparations of HIV-1_{HZ321} (density gradient- and chromatography-purified virus prepared as described previously [27, 34]).

Protein kinase activities are activated by autophosphorylation, transphosphorylation, or dephosphorylation (1). In our system, phosphatase inhibitors were present in the phosphorylation assay mix. Activation of the virus-associated protein kinase might be triggered by autophosphorylation. An in-gel phosphorylation assay was performed as previously described (19) with the HIV preparation to detect possible autophosphorylation of the protein kinase. As shown in Fig. 2A, three proteins were autophosphorylated. Two of them, corresponding to 53K and 42K, were visualized previously, and the third one corresponded to a 62K band. Although this last product was consistently ^{32}P labelled in in-gel assays, it did not appear on every SDS-PAGE gel of kinase assays (Fig. 1A and 2B). Proteins of the same molecular mass were autophosphorylated in the cell lysate, indicating that they might correspond to cellular kinases. Thus, Fig. 2A shows that the kinase activity is consistently provided by two proteins, 53K and 43K. In order to determine whether these two proteins were linked by a disulfide bond, a phosphorylation assay with lysed virus was performed as described in the legend to Fig. 1A, stopped by adding 15 μl of 5× Laemmli sample buffer (without any 2-mercaptoethanol) supplemented or not with 100 mM dithiothreitol. As shown in Fig. 2B, no differences were detected in the phosphorylation pattern under reducing and nonreducing conditions, indicating that the two autophosphorylated proteins are either two different proteins or two distinct subunits of a single enzyme.

To identify the virus-associated protein kinases, phosphorylated proteins were separated by high-resolution SDS–10% PAGE (acrylamide/bisacrylamide ratio, 30:0.4), transferred to a membrane, and immunodetected with an antiserum against ERK1 and ERK2 mitogen-activated protein kinase (MAPK) proteins. As shown in Fig. 3A, ERK2 MAPK is the only isoform detected in viral preparations, whereas antibodies bind to ERK1 and ERK2 isoforms in cell lysates. It is noteworthy that the unphosphorylated and phosphorylated forms of both ERK1 and ERK2 are detected in the cell lysate but do not migrate at the same level. This is due to differences in the molecular weights of the unphosphorylated and phosphorylated forms of MAPK (5). Thus, the only form of ERK2 detected in the viral lysate is the unphosphorylated one, the most abundant form of this protein. The 53K phosphorylated protein could not be identified but corresponds to a second protein kinase associated with virus particles. To confirm that ERK is indeed associated with HIV-1 virions, Western blot analysis was performed with banded radiophosphorylated virus preparations collected from a continuous sucrose density gradient. The anti-MAPK antiserum revealed ERK2 in virus-enriched fractions monitored by density (virus gravity, 1.18 to 1.17 g/ml [fractions 9 and 10]) and by immunodetection with an anti-CA antibody which detected mature $p24^{Gag}$ and unprocessed $p55^{Gag}$ (Fig. 3B). A ^{32}P exposure of the same membrane revealed the expected phosphorylated proteins, 53K and 43K (Fig. 3B), as well as 24K, 17K, and 15K (Fig. 1A). To rule out the possibility that ERK2 associates with cellular vesicular materials which happen to have a density similar to that of retrovirus particles, supernatant from uninfected cells was processed as described above (centrifuged over a linear gradient of sucrose, followed by a kinase assay and Western blotting). No ERK2 was detected with the anti-MAPK antiserum in any fraction, although a ^{32}P exposure revealed a phosphorylated band of high molecular weight (91K) at low density (1.12 to 1.13 g/ml) (data not shown). These results show that ERK2 MAPK is associated with virus particles and is not a cellular contaminant.

To determine whether ERK2 MAPK is strongly associated with retrovirus particles, the phosphorylation profiles of viruses prepared from HIV-1_{HZ321}-infected HUT78 T cells (as before), HIV-1_{RF}-infected CEM T cells, HIV-1_{LAI}-infected U937 cells (promonocytic cell line), and human T-cell leukemia virus type 1 (HTLV-1)-producing C91PL T cells were compared. In vitro kinase assays were performed with these viral preparations in three independent experiments. An analysis of phosphorylated proteins showed similar patterns for HIV-1_{RF} and HIV-1_{HZ321} (Fig. 4A, lanes a and b). Moreover, HIV-1_{LAI} derived from a promonocytic cell line, as well as HTLV-1 virions, showed phosphorylated proteins, suggesting the presence of virus-associated protein kinases in these viral preparations (Fig. 4A, lanes c and d). However, the phosphorylation pattern is not identical to the one obtained with HIV-1 strains derived from T-cell lines (Fig. 4A, lanes a and b). Finally, Fig. 4 shows that ERK2 MAPK was associated with all the viral preparations used. Note that with HIV-1_{RF}, an additional band was revealed by anti-MAPK. Whether this protein corresponds to ERK1 needs to be determined with a specific antibody. Thus, these results indicate that ERK2 is strongly

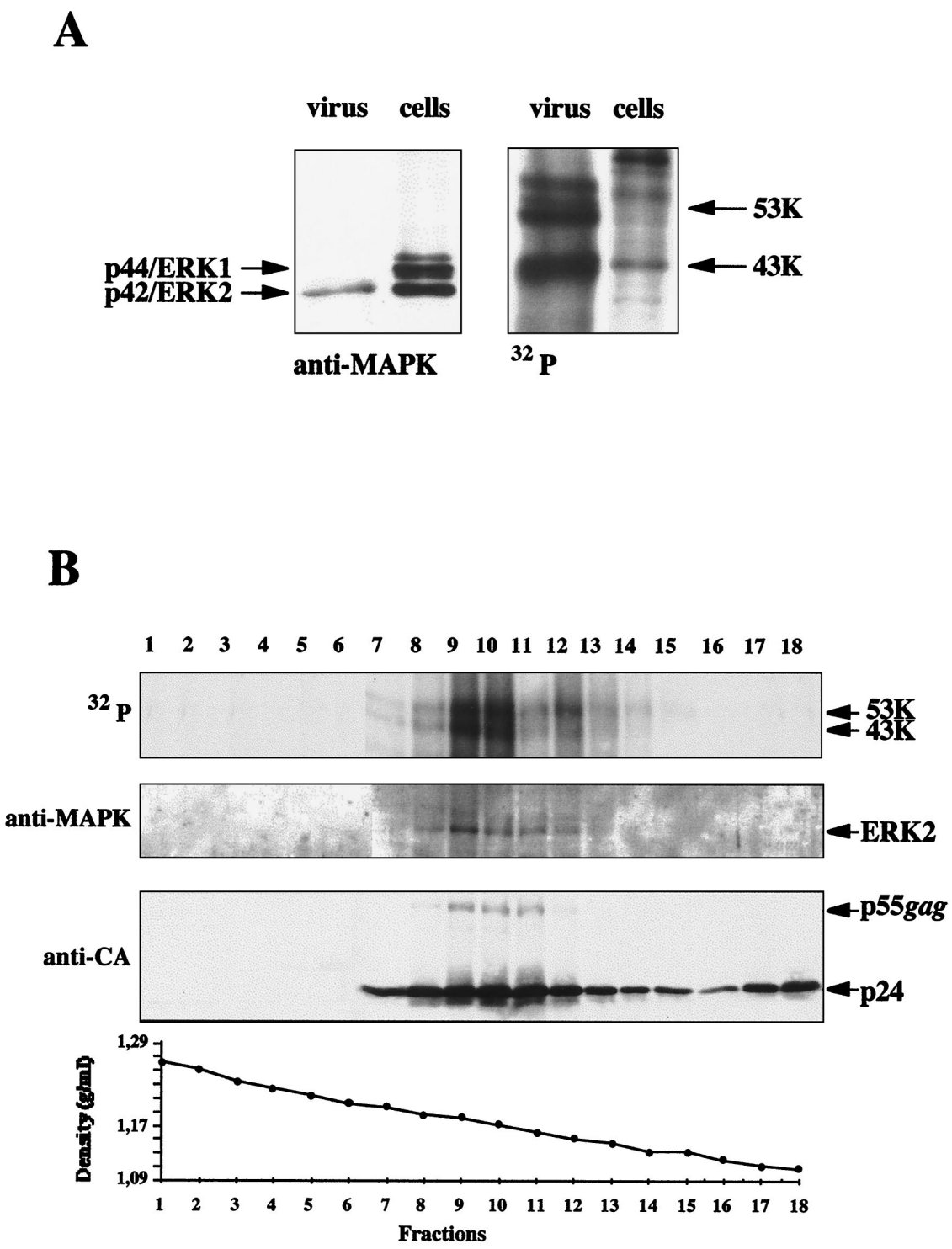

FIG. 3. Detection of ERK2 MAPK protein within radiophosphorylated HIV-1 particles. (A) Immunodetection of radiophosphorylated ERK2 MAPK protein within virus particles by SDS–10% PAGE (acrylamide/bisacrylamide ratio, 30:0.4) of the radiophosphorylated virus (50 μg) and infected-cell lysate (20 μg), transferred to a polyvinylidene difluoride (PVDF) membrane, with an antiserum against the ERK1 (p44) and ERK2 (p42) MAPK proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). (B) Sucrose density gradient analysis of HIV-1 virions. HIV-1_{HZ321} virions produced in HUT78 cells were purified over a linear gradient (20 to 60% [wt/vol]) of sucrose and centrifuged for 18 h at 25,000 rpm and 4°C in an SW41 rotor (Beckman). Eighteen fractions of 0.6 ml were collected from the top of each tube by the fluorinert method. Fractions were analyzed for density with a refractometer. Each fraction was diluted with phosphate-buffered saline, pelleted in a 50.3Ti rotor (45,000 rpm, 1 h, 4°C), and solubilized in 50 μl of lysis buffer. Kinase assays were performed with each sample, and proteins were separated by SDS–12% PAGE, transferred to PVDF membranes, and autoradiographed. The membranes were incubated with anti-MAPK antiserum, stripped (stripping buffer: 62.5 mM Tris-HCl [pH 6.7], 100 mM 2-mercaptoethanol, 2% SDS; 30 min; 50°C), and incubated with a human monoclonal anti-CA antibody (kindly provided by D. Bourel, CTS, Lille, France) prior to chemiluminescense revelation (Amersham).

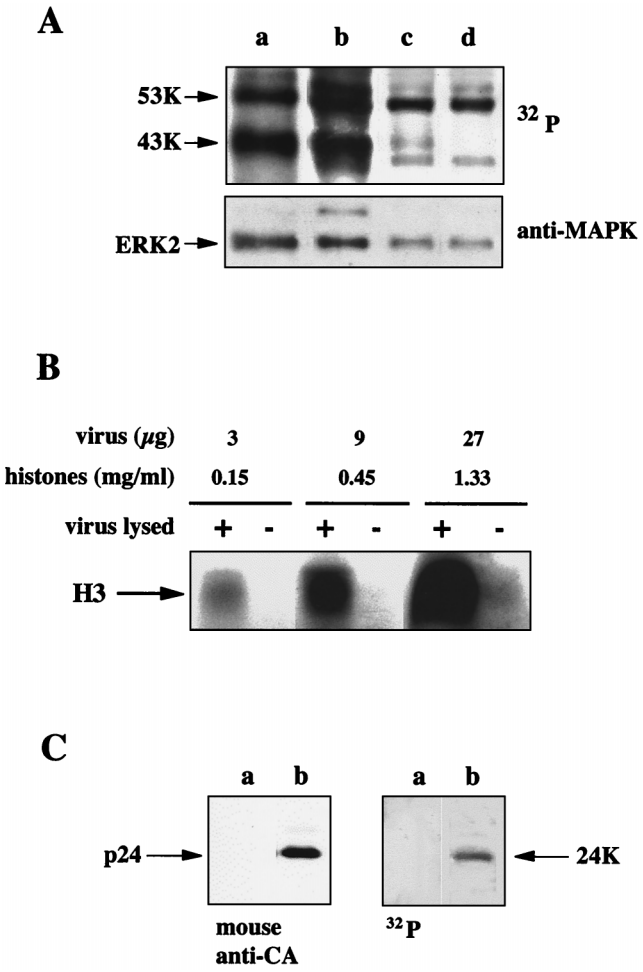

FIG. 4. (A) Retrovirus-associated protein kinase activity and ERK2 MAPK detection. HIV-1_{HZ321} (a) was purified with multiple sucrose density gradients (15). Cell-free supernatants (50 ml; clarified by being filtered through a 0.45-μm-pore-size filter) from HIV-1_{RF}-infected CEM cells (b), HIV-1_{LAI}-infected U937 cells (c), and HTLV-1-infected C91PL cells (d) were ultracentrifuged through a 20% sucrose cushion (SW28 rotor, 23,000 rpm, 3 h, 4°C), and pelleted virions were solubilized in 50 μl of lysis buffer. Seventy micrograms of each viral lysate was used for kinase assays. Proteins were separated by SDS–10% PAGE, transferred to a PVDF membrane, and autoradiographed or incubated with anti-MAPK antiserum prior to chemiluminescense revelation. (B) Phosphorylation of histone H3 by the virus-associated protein kinase activity. The phosphorylation assay was performed with either 0.5% Nonidet P-40-lysed virus (+) or unlysed virus (−) in kinase buffer containing a mix of histones as exogenous substrates (Boehringer, Mannheim, Germany). The autoradiograph shows the phosphorylation of histone H3 in reaction mixtures containing increasing amounts of virus and histones. (C) Immunoprecipitation of the radiophosphorylated CA protein. Radiophosphorylated virus (100 μg) was immunoprecipitated with an irrelevant human immunoglobulin G (a) or human anti-CA antibody (b), separated by SDS–13% PAGE, transferred to a PVDF membrane, and autoradiographed or revealed by a mouse monoclonal antibody against the Gag CA protein (kindly provided by B. Verrier, UMR, Lyon, France) prior to chemiluminescense revelation.

associated with HIV and HTLV-1 particles in a cell-independent manner.

To further demonstrate that the protein kinases were incorporated into the virus, a phosphorylation assay was performed with intact virus in the presence of a mix of histones as substrates. Figure 4B shows that no phosphorylation of the major H3 histone protein by intact virus occurred, whereas phosphorylation of H3 was detected when the virus had previously been lysed. These results demonstrate that the kinase proteins were indeed incorporated inside virus particles and were not associated with the external side of the viral membrane.

To determine whether Gag CA was phosphorylated, immunoprecipitation with the anti-CA protein of the radiophosphorylated virus was carried out. Immunoprecipitated Gag CA was ^{32}P labelled, showing that Gag CA is indeed phosphorylated (Fig. 4C). Furthermore, automated solid-phase sequencing by Edman degradation of the 24K protein revealed that at this molecular weight, the product was exclusively the Gag CA viral protein (data not shown). The other viral components known to be present in virions, such as reverse transcriptase, integrase, Vpr, gp120, and gp41, do not correspond to phosphorylated proteins (data not shown). As shown in Fig. 1A, a phosphorylated protein, 17K, present within radiophosphorylated virus, superimposed with an anti-matrix (MA; p17) antibody (data not shown). The Gag MA might be phosphorylated, but immunoprecipitation assays cannot yet formally determine whether the Gag MA is phosphorylated (data not shown).

Recently, Bukrinskaya et al. (4) showed the presence of a serine protein kinase selectively incorporated into virus particles. Our findings confirm the presence of a virus-associated protein kinase activity and demonstrate that HIV-1 particles incorporate two protein kinases, one of which is ERK2 MAPK. Furthermore, we have shown that ERK2 MAPK is associated with HIV-1 particles produced by T-cell lines and one promonocytic cell line, as well as with HTLV-1 particles. Thus, the strong association of ERK2 with retrovirus particles suggests an important role for this protein during the retroviral life cycle. The MAPK signal transduction pathway represents an important mechanism by which growth factors, hormones, and neurotransmitters regulate physiological responses (24, 30). The consensus sequence of MAPK is represented by the P-X-S/T-P motif or by the S/T-P minimal sequence (7). This minimal sequence can be found in 20 HIV isolate sequences of the Gag CA protein, and two isolates contain a P-X-S/T-P sequence at the MAPK phosphorylation site (8, 18). These observations suggest that the Gag CA protein is a substrate of MAPK. However, whether Gag CA is an ERK2 substrate still needs to be determined.

HIV infection modulates T-cell activation, although little is known about the activation message propagated from the cell membrane to the nucleus (6, 10, 11). Phosphorylation of Gag MA on serine and tyrosine residues in infected cells appears to be required for the translocation of viral nucleic acids to the nucleus (4, 13, 14, 31). More specifically, Bukrinskaya et al. (4) showed that Gag MA protein was the only viral structural protein phosphorylated into virions. We demonstrate that in addition to Gag MA, Gag CA can be phosphorylated by one of the kinases incorporated into virions. These results are being extended to determine whether there is an active role for the phosphorylation of viral proteins by serine/threonine kinases incorporated into HIV-1 virions in the viral life cycle.

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