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The Late-Domain-Containing Protein p6 Is the Predominant Phosphoprotein of Human Immunodeficiency Virus Type 1 Particles

Barbara Müller,[1]* Tilo Patschinsky,[2] and Hans-Georg Kräusslich[1]

Abteilung Virologie, Universitätsklinikum Heidelberg, D-69120 Heidelberg,[1] and Heinrich-Pette-Institut an der Universität Hamburg, D-20251 Hamburg,[2] Germany

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The Gag-derived protein p6 of human immunodeficiency virus type 1 (HIV-1) plays a crucial role in the release of virions from the membranes of infected cells. It is presumed that p6 and functionally related proteins from other viruses act as adapters, recruiting cellular factors to the budding site. This interaction is mediated by so-called late domains within the viral proteins. Previous studies had suggested that virus release from the plasma membrane shares elements with the cellular endocytosis machinery. Since protein phosphorylation is known to be a regulatory mechanism in these processes, we have investigated the phosphorylation of HIV-1 structural proteins. Here we show that p6 is the major phosphoprotein of HIV-1 particles. After metabolic labeling of infected cells with [*ortho*-^{32}P]phosphate, we found that phosphorylated p6 from infected cells and from virus particles consisted of several forms, suggesting differential phosphorylation at multiple sites. Apparently, phosphorylation occurred shortly before or after the release of p6 from Gag and involved only a minor fraction of the total virion-associated p6 molecules. Phosphoamino acid analysis indicated phosphorylation at Ser and Thr, as well as a trace of Tyr phosphorylation, supporting the conclusion that multiple phosphorylation events do occur. In vitro experiments using purified virus revealed that endogenous or exogenously added p6 was efficiently phosphorylated by virion-associated cellular kinase(s). Inhibition experiments suggested that a cyclin-dependent kinase or a related kinase, most likely ERK2, was involved in p6 phosphorylation by virion-associated enzymes.

The *gag* gene of human immunodeficiency virus type 1 (HIV-1) encodes all functional domains required for the assembly and release of enveloped virus-like particles (for a review, see reference 54). In the infected cell, Gag is synthesized as a 55-kDa polyprotein (Pr55^{Gag}) and transported to the plasma membrane, where it assembles into spherical immature particles. Concomitant with or after the release of particles from the host cell by budding, the virus-encoded protease (PR) cleaves Gag into its functional subdomains, matrix (MA), capsid (CA), nucleocapsid (NC), and p6. This proteolytic maturation results in a structural rearrangement of Gag subunits within the particle and is required for virus infectivity. In the mature virion, NC condenses the viral RNA, whereas CA forms a conical shell encasing the nucleocapsid, and MA forms a protein layer underneath the virion envelope.

The C-terminal p6 domain of Gag contains the so-called late domain of HIV, a sequence which has been found to be required for the efficient separation of the virus envelope and the cell membrane. Mutations impairing p6 function result in accumulation of late budding structures at the cell surface. Particles typically stay connected to the cell membrane by thin membranous stalks, and often chain- or tree-like structures of budding particles connected to each other are observed. The virions that are released display mostly immature morphology, and infectivity is severely reduced. In addition, a decrease in the amounts of *pol* products packaged into the particles has been reported (18, 27, 65). The presence of a functional late domain in Gag has been demonstrated for many retroviruses (18, 41, 43, 61, 64, 66), but analogous domains have also been detected in unrelated viruses, like vesicular stomatitis virus (VSV), Ebola virus, and rabies virus (12, 21, 22). Mutational analyses and sequence comparisons have identified three distinct sequence motifs crucial for late-domain function, namely, PT/SAP for HIV-1 and related lentiviruses (18, 27), YXXL for equine infectious anemia virus (43), and PPXY—frequently in conjunction with PT/SAP—for other retroviruses (62, 64, 66), as well as for unrelated viruses (21, 22, 29).

Although the exact mechanism of late-domain function is not known, it is assumed that late-domain-containing proteins act as molecular adapters recruiting cellular factors involved in virus-cell separation to the budding site and that the conserved core motifs serve as docking sites for those cellular factors. Consistent with this, retroviral late domains can function independently of their positions within the structural protein (41, 62) and are functionally interchangeable irrespective of their amino acid sequences (12, 41, 67), and the severity of the effects of mutations within the late domain varies with the type of host cell used for analysis (14, 48). Whereas the molecular events governing virus-cell separation are not well understood, the experimental evidence currently available points to components of the cellular protein-sorting and endocytosis machineries being involved in virus release. Late domains of different viruses have been shown to interact via their PPXY motifs with the WW domain containing ubiquitin ligase Nedd4, which plays a role in endocytotic internalization of

* Corresponding author. Mailing address: Abteilung Virologie, Universitätsklinikum Heidelberg, Im Neuenheimer Feld 324, D-69120 Heidelberg, Germany. Phone: 49-6221-561325. Fax: 49-6221-565003. E-mail: Barbara_Mueller@med.uni-heidelberg.de.

cellular membrane proteins, to bind to Tsg101, which is implicated in endosomal sorting, or to recruit AP-2, which is involved in clathrin-mediated endocytosis (22, 44, 56).

It appears likely that the complex process of virus budding is tightly regulated to ensure timely and accurate release of the assembled virion. Such regulation may be accomplished by posttranslational modification of late-domain proteins. HIV-1 and simian immunodeficiency virus p6 proteins, as well as the functionally related phosphoprotein pp12 from Moloney murine leukemia virus, have been shown to be partially monoubiquitinylated in the virion (38), and whereas ubiquitinylation of p6 itself does not seem to be essential (40), several lines of evidence indicate that the presence of ubiquitin moieties at the site of budding, as well as the interaction of late-domain proteins with ubiquitin binding proteins, is important for virus release (22, 42, 47, 52). In the regulation of membrane protein endocytosis, ubiquitinylation and protein phosphorylation are often used in a stepwise manner, where phosphorylation enhances subsequent ubiquitinylation (see reference 25 for a review). Interestingly, the cellular membrane proteins amiloride-sensitive sodium channel (ENaC) and connexins 43 and 45, which share sequence elements with viral late-domain proteins, are also regulated by both types of posttranslational modification. These proteins display rapid turnover at the membrane regulated by ubiquitin-mediated processes (26, 30) and are phosphorylated by a number of kinases which regulate their activities, presumably in part by influencing protein levels at the membrane (31, 51).

The phosphorylation of virus-encoded proteins has been studied in many systems, and several viral proteins harboring late domains have been described as phosphoproteins (see Discussion). However, the potential correlation between phosphorylation and late-domain function has not been analyzed. In the case of HIV-1, studies from many laboratories have provided information concerning phosphorylation of individual viral proteins. Early biochemical analyses of the *gag* gene products of HIV-1 revealed phosphorylated forms of the MA and CA structural proteins (35, 55), and possible functional implications of their phosphorylation have been discussed (4, 7, 16, 17, 28). In addition, phosphorylation of the HIV-1 accessory proteins Vpu (53), Nef (10, 19), Rev (11, 23), Vif (63), Tat (33), and Vpr (36, 68) has been reported. However, these studies were focused on one particular viral protein and often involved the investigation of protein phosphorylation in vitro or upon overexpression, whereas a comprehensive analysis of HIV-1 protein phosphorylation in virions and infected cells has not been reported. As part of our studies of HIV-1 protein modification, we had carried out metabolic labeling of HIV-1-infected cells using [*ortho*-^{32}P]phosphate. When we analyzed the labeled material, we found that the majority of virion-associated phosphoproteins could be immunoprecipitated with antisera against p6, which until now has not been recognized as a phosphoprotein. This initial observation, together with the possibility that this modification has functional implications, prompted us to examine the phosphorylation of HIV-1 p6. We present here a detailed characterization of p6 phosphorylation showing that p6 is partially modified at Ser, Thr, and Tyr residues in infected cells, can serve as a substrate for virion-associated kinases, and represents the major phosphorylated viral protein in purified HIV-1 particles.

MATERIALS AND METHODS

Metabolic labeling of HIV-1-infected cells. Metabolic labeling of HIV-1 NL4-3-infected MT-4 cells with [^{35}S]cysteine was carried out as described previously (36). For metabolic labeling with [^{32}P]phosphate, MT-4 cells were infected with HIV-1 NL4-3 by coculture as described previously (60). At 18 or 24 h postinfection, the cells were starved for 2 h in phosphate-free RPMI medium. Subsequently, they were incubated in RPMI medium containing 0.5 mCi of [*ortho*-^{32}P]phosphate per ml. Following incubation for another 12 h, the cells were harvested, and virus was prepared from the tissue culture supernatant by centrifugation through an OptiPrep density gradient as described by Dettenhofer and Yu (15). Virus was concentrated by being pelleted through a 20% (wt/vol) sucrose cushion and was resuspended in Tris-buffered saline and immediately analyzed by immunoprecipitation or stored for up to 2 weeks at −80°C.

For the labeling experiments carried out in the presence of inhibitors, MT-4 cells were treated as described above. The phosphatase inhibitor cocktail (final concentrations, 250 pM cypermethrin, 50 nM ocadaic acid, 50 nM tautomycin, and 50 μM dephostatin; all from Calbiochem) was added to the medium concomitant with the addition of label. Treatment with the HIV PR inhibitor saquinavir (45) (final concentration, 2.5 μM) started 3 h before labeling was initiated. Inhibitors were present in all cases until the cells were harvested. After being labeled for 2 or 12 h, the cells were harvested and lysed in RIPA buffer containing broad-range protease inhibitors (see below) and the phosphatase inhibitor cocktail. Cell extracts were subjected to immunoprecipitation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by phosphorimage analysis as described below.

Subtilisin treatment of virions. Unlabeled virus was concentrated from the tissue culture supernatant by centrifugation through a 20% sucrose cushion. The virus pellet was resuspended in 750 μl of phosphate-buffered saline, yielding a total protein concentration of approximately 2 mg/ml. Eighty microliters of subtilisin (Roche; 10 mg/ml in phosphate-buffered saline) was added, and the mixture was incubated for 90 min at 30°C. The enzyme was inactivated by the addition of 10 μl of PefablocSC (200 mM). Subsequently, the virus was purified by velocity centrifugation through an OptiPrep gradient (15). SDS-PAGE was performed to control for efficient digestion of external protein, as monitored by the disappearance of serum albumin contamination, as well as for the integrity of internal virion proteins.

Immunoprecipitations. Cells or virus samples were lysed in RIPA buffer (20) containing an inhibitor cocktail (final concentrations, 2 mM PefablocSC, 10 μM E-64, 1 μM pepstatin, and 1 mM sodium orthovanadate) for 30 min on ice. Cell lysates were cleared by brief centrifugation and pretreated by immunoprecipitation using a mixture of three rabbit preimmune sera. For precipitation, we used antisera prepared against recombinant HIV-1-encoded proteins or a p6-derived peptide (5). Immunoprecipitation was carried out using standard procedures (20) with protein A-Sepharose (Amersham Pharmacia), and immunoprecipitates were resolved by SDS-PAGE. Following electrophoresis, the gels were stained with Coomassie blue to detect marker bands, dried, and subjected to phosphorimage analysis using a FujiBAS2000 imager.

In vitro phosphorylation by virion-associated kinases. HIV-1 NL4-3 was grown in MT-4 cells or HeLaP4 cells and purified as described above. Figure 1, lane 1, illustrates the purity of the samples obtained in this way. Virus concentrate was stored in aliquots at −80°C and thawed immediately before use. Ten microliters of virus suspension (corresponding to approximately 5 μg of CA) was mixed with 15 μl of lysis buffer (0.75% Nonidet P-40, 50 mM HEPES, pH 7.5), and the mixture was incubated on ice for 30 min. Labeling was then initiated by adding the lysate to 100 μl of reaction mixture. Unless otherwise stated, the final buffer conditions were 50 mM HEPES (pH 7.5), 5 mM $MnCl_2$, and 10 μCi of [γ-^{32}P]ATP. The reaction mixtures were incubated at 30°C for 20 to 40 min and quenched by the addition of 25 mM EDTA. Samples were either directly subjected to SDS-PAGE or immunoprecipitated as described above. The samples were separated using the indicated SDS-PAGE systems and analyzed by phosphorimaging.

Phosphorylation of exogenously added proteins. HIV-1-encoded proteins were expressed as recombinant proteins in *Escherichia coli* either without additional sequences and purified by conventional chromatography (CA, reverse transcriptase [RT] heterodimer, or integrase [IN]) or as His-tagged or glutathione *S*-transferase (GST) fusion proteins (MA and p6) purified using a Ni^{2+}-chelate column or glutathione-Sepharose according to the manufacturer's instructions (Qiagen Expressionist; Amerham Pharmacia manual). Purified protein (1.5 μg each) was added to 15 μl of an in vitro phosphorylation mixture (corresponding to 1.25 μl of virus suspension) prepared as described above, and samples were incubated for 40 min at 25°C. The reactions were quenched by the

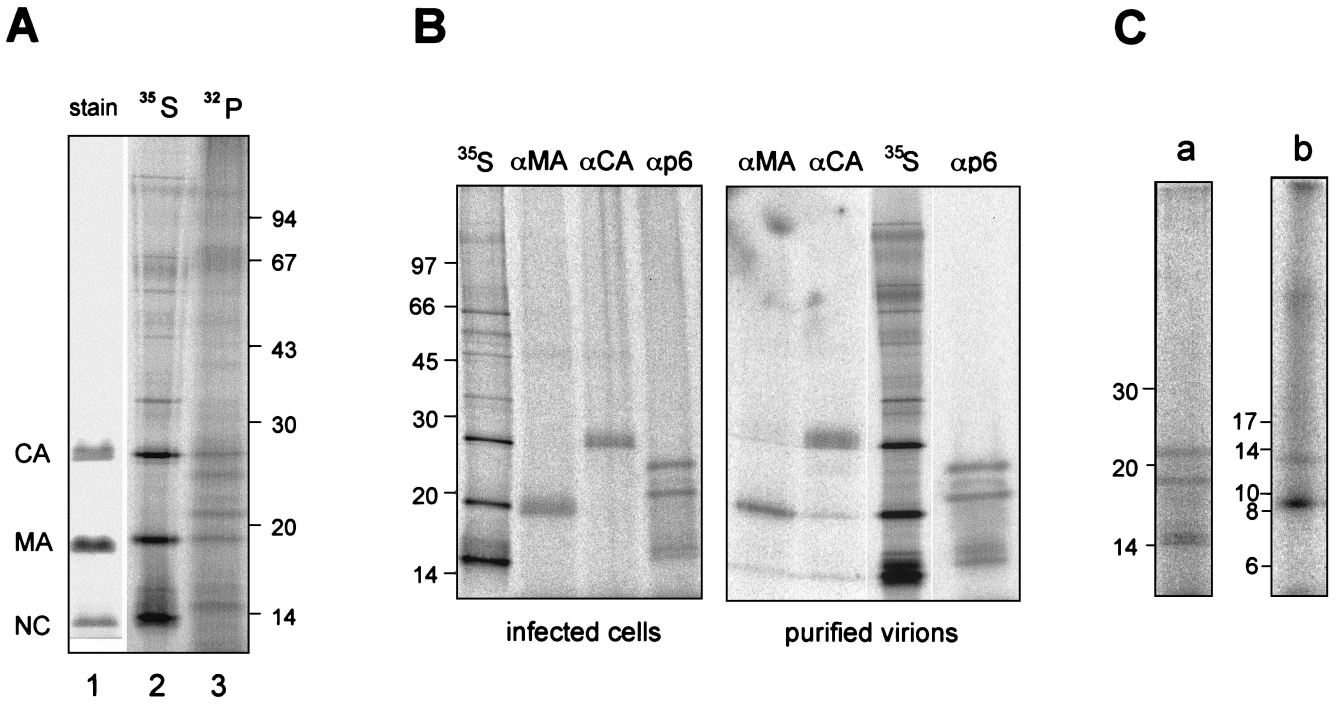

FIG. 1. (A) Metabolic labeling of virion-associated proteins. Virus particles labeled with ^{35}S or ^{32}P were prepared and purified as described under Materials and Methods. Samples were separated by SDS-PAGE (17.5% polyacrylamide; cross-linking ratio, 200:1). Labeled proteins were detected by phosphorimage analysis (lanes 2 and 3). Lane 1 shows a virus sample from a ^{35}S labeling experiment visualized by silver staining to determine the purity of the sample. The positions of the major structural proteins are indicated on the left; the molecular masses of marker proteins (in kilodaltons) are shown on the right. (B) Identification of phosphorylated-p6-related proteins by immunoprecipitation. HIV-infected cells were metabolically labeled with [*ortho*-^{32}P]phosphate for 12 h as described under Materials and Methods. Lysates from 3×10^6 infected cells, as well as from purified virions corresponding to 2 ml of tissue culture supernatant, were subjected to immunoprecipitation according to standard procedures using polyclonal antisera prepared against the indicated HIV proteins. The immunoprecipitates were separated by SDS-PAGE (17.5% polyacrylamide; cross-linking ratio, 200:1), and radiolabeled proteins were detected by phosphorimage analysis. Purified virus particles labeled with [^{35}S]cysteine were applied to the gel as a reference (lanes ^{35}S). (C) Direct comparison of the relative mobilities of p6-related bands using two different PAGE systems. Cell extract harvested 12 h after the initiation of labeling was subjected to immmunoprecipitation using polyclonal antiserum prepared against p6. The sample was split into two halves and separated by SDS-PAGE on a Tris-glycine-SDS gel (17.5% polyacrylamide; cross-linking ratio, 200:1) (a) and a Tris-Tricine-SDS gel (46) (16.5% polyacrylamide; cross-linking ratio, 33:1) (b). The positions of marker proteins are indicated to the left of each lane, with their molecular masses given in kilodaltons.

addition of 25 mM EDTA, and samples were analyzed directly by SDS-PAGE and phosphorimage analysis.

Phosphoamino acid analysis. Virus was metabolically labeled with [*ortho*-^{32}P]phosphate and purified as described above. Fifteen microliters of purified virus lysate (corresponding to ca. 15 ml of tissue culture supernatant) was subjected to immunoprecipitation using αp6 antiserum. The immunoprecipitate was resolved by SDS-PAGE and transferred to an Immobilon P membrane by blotting according to the manufacturer's instructions. Labeled bands were identified by exposure of the membrane to X-ray film (Kodak XAR), and portions of the membrane corresponding to the radioactive areas were cut out for further analysis. Membrane-bound protein was hydrolyzed, and two-dimensional electrophoresis was performed essentially as described by Boyle et al. (2).

RESULTS

Phosphorylation of HIV-1 p6 in infected cells and virus preparations. To investigate phosphorylation of HIV-1 proteins in infected cells and virus preparations, we carried out metabolic labeling of HIV-1 (strain NL4-3)-infected MT-4 cells using [*ortho*-^{32}P]phosphate. After 12 h of labeling, a lysate from the cells was prepared, and virions were purified from the concentrated tissue culture supernatant by centrifugation through an OptiPrep velocity gradient. Purified virus from two independent labeling experiments separated by SDS-PAGE showed a distinctive pattern of bands upon phosphorimage analysis (Fig. 1A, lane 3). For comparison, [^{35}S]cysteine-labeled virus purified by the same procedure was applied to the same gel (Fig. 1A, lane 2), and a silver stain of this preparation is also shown to demonstrate virus purity (Fig. 1A, lane 1). The main bands visible in the [^{35}S]cysteine-labeled preparation are the virion structural proteins MA, CA, and NC, indicating the high purity of the gradient-purified samples. Additional labeled bands can be attributed to other viral proteins (IN, RT, and viral glycoproteins). It should be noted that p6 itself, due to the lack of cysteine or methionine residues, is not detectable in ^{35}S-labeled preparations.

Side-by-side comparison revealed that two of the main phosphorylated bands in HIV-1 particles comigrated with ^{35}S-labeled MA and CA, but the remaining major bands could not be associated with any virion protein according to their electrophoretic mobilities. To identify phosphorylated viral proteins, labeled cell lysates, as well as purified virus, were subjected to immunoprecipitation with specific antisera raised against recombinant HIV-1 proteins. Using this approach, we detected phosphorylated forms of the Gag proteins MA and CA (Fig. 1B) and of the accessory proteins Vpu and Vpr (reference 36 and data not shown) in the cell lysate. Phosphor-

ylated MA (pMA) and pCA, as well as pVpr, were also precipitated from purified virions (Fig. 1B) (36). Phosphorylation of MA, CA, Vpr, and Vpu is in agreement with previously published reports (35, 36, 53, 55, 68). The most striking result was obtained, however, when antiserum against the p6 protein was used for immunoprecipitation (Fig. 1B). A distinctive pattern of seven to eight bands with apparent molecular masses between 14 and 22 kDa was observed in immunoprecipitates from infected cells and from virions. The proteins from the αp6 immunoprecipitate represented essentially all major bands from the total virus lysate with the exception of those comigrating with MA and CA (compare Fig. 1A and B).

To confirm this result, we carried out additional immunoprecipitations using virus and cell lysates from two independent metabolic labeling experiments and using αp6 antisera from different sources raised against a peptide antigen or against a GST-p6 fusion protein (not shown). Although there were slight variations in relative band intensities, the pattern shown in Fig. 1B was reproducibly detected in all experiments. Because no labeled bands were detected in αp6 immunoprecipitates from ^{32}P-labeled extracts of uninfected MT-4 cells (not shown), we concluded that all of the labeled forms shown in Fig. 1B corresponded to p6 isoforms. A corresponding band pattern was not detectable in αp6 immunoblots of unlabeled virus (not shown), indicating that the modification involves only a minor fraction of p6 molecules. From two-dimensional gel analyses of purified virus, we estimate that αp6-reactive spots which would match phosphorylated forms of free p6 according to apparent molecular mass and isoelectric point represent at most 5% of the protein (data not shown).

The apparent molecular masses of the labeled proteins estimated from their electrophoretic mobilities in SDS-PAGE (ca. 14 to 22 kDa) differed significantly from the calculated molecular mass of p6 (5.8 kDa). However, it is known that phosphorylation can change the migration behavior of proteins in SDS-PAGE in a manner not proportional to the actual increase in molecular mass. In addition, we noted that the low-cross-linking polyacrylamide gels used in these experiments tended to increase aberrant electrophoretic mobility caused by the particular properties of a protein (for instance, the position of the 7-kDa NC protein, which migrates close to the 14-kDa marker protein on these gels [Fig. 1A, lanes 1 and 2]). To determine whether the electrophoretic mobilities of the phosphorylated p6 (pp6) forms was due to this property of the gel or reflected the actual molecular masses of the modified forms, aliquots from a single immunoprecipitation experiment were analyzed on different gels. As shown in Fig. 1C, separation on a gel with a high cross-linking ratio designed for the analysis of small proteins (46) resulted in a concentration of most of the labeled pp6 bands at a molecular mass close to that of the unmodified p6 protein, while the typical pattern was observed for the same sample on a low-cross-link gel. The total intensity of the two distinct bands visible in lane b (935 relative units) corresponded to the sum of band intensities from lane a (898 relative units). We therefore concluded that the high apparent molecular masses of most pp6 forms were due to modification of the net charge by hyperphosphorylation. The band migrating at a position corresponding to 12 kDa was repeatedly observed in αp6 immunoprecipitates on Tris-Tricine-SDS gels (Fig. 1C, lane b). According to its apparent molecular weight, this band could represent a monoubiquitinylated form of pp6. However, attempts to test this by immunoprecipitation of labeled virus extracts using antiserum against ubiquitin did not yield detectable signals (data not shown).

p6 is phosphorylated at Ser, Thr, and Tyr residues. As shown above, several forms of pp6 with different apparent molecular masses were observed, indicating that p6 is differentially phosphorylated at several residues. The p6 protein of the HIV-1_{NL4-3} strain, which was used in these experiments, contains 13 residues which could theoretically be phosphorylated (eight Ser, four Thr, and one Tyr [Fig. 2A]). In order to identify phosphorylated residues in pp6, phosphoamino acid analysis was performed on αp6 immunoprecipitates from metabolically labeled purified virus. Due to the low radioactivity of the sample obtained after precipitation, electrophoresis, and blotting, bands corresponding to the entire αp6 immunoprecipitate were subjected to phosphoamino acid analysis. We detected a strong signal for pSer and a weaker signal for pThr (Fig. 2B). Furthermore, a faint spot migrating at the position of pTyr was also observed (Fig. 2B), indicating that pp6 is phosphorylated at Ser, Thr, and Tyr residues and may be phosphorylated at multiple Ser and Thr residues. It should be noted that due to the differential instabilities of phosphoamino acids against acid hydrolysis, the intensity of the spots does not quantitatively reflect the ratio of phospho-Ser, -Thr, and -Tyr in the sample. Due to the low activity of the recovered material, phosphoamino acid analysis of individual pp6 isoforms with different electrophoretic mobilities was not feasible.

Phosphorylation of p6 occurs concomitant with or after proteolytic processing of Gag. In order to determine at which point in the HIV-1 life cycle p6 phosphorylation occurred, we performed metabolic labeling for different periods of time and in the presence of inhibitors. As outlined above, the HIV p6 protein is synthesized as part of the $Pr55^{Gag}$ polyprotein precursor, which assembles at the plasma membrane. Processing of the precursor by the virus-encoded PR, releasing p6 as well as the other functional Gag subunits, occurs concomitant with virus assembly or budding.

The possibility that phosphorylation is carried out solely by virion-associated kinases inside the mature particle can be ruled out by the observation that immunoprecipitation from the lysate of infected cells yielded a very similar pattern of pp6 forms as immunoprecipitation from virus lysate (Fig. 1B). To determine whether modification occurred before or after the release of p6 from the Gag precursor, a metabolic labeling experiment analogous to the one described above was carried out in the presence of 2.5 μM saquinavir (45), a specific inhibitor of HIV-1 PR which prevents processing of the Gag precursor. In addition, we performed metabolic labeling for shorter periods of time (2 h) and in the presence of a cocktail of cell-permeable phosphatase inhibitors to maximize recovery of pp6 forms. p6-derived proteins were collected by immunoprecipitation from cell lysates and analyzed by SDS-PAGE and phosphorimaging. As shown in Fig. 3, metabolic labeling for 2 h in the absence of saquinavir yielded only faster-migrating isoforms of pp6 (lane 1), while the slower-migrating bands were much more prominent in the presence of phosphatase inhibitors (lane 3). This result is consistent with our conclusion that these bands are derived from hyperphosphorylation. In

A

LQSRPEPTAPPEESFRFGEETTTPSQKQEPIDKELYPLASLRSLFGSDPSSQ

B

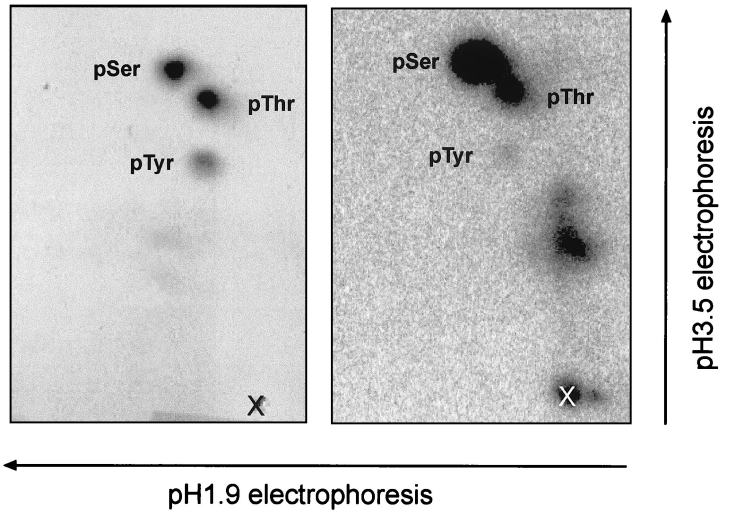

FIG. 2. (A) Amino acid sequence of p6 of HIV-1_{NL4-3}. Ser, Thr, and Tyr residues are in boldface. (B) Phosphoamino acid analysis of pp6. p6 was immunoprecipitated from metabolically labeled virus lysate and subjected to phosphoamino acid analysis as described under Materials and Methods. On the right is the autoradiogram; on the left are shown the stained marker phosphoamino acids separated in parallel. An X indicates the origin. The labeled material between the origin and the phosphoamino acid spots in panel b resulted from partial hydrolysis of the protein.

the presence of the PR inhibitor saquinavir (Fig. 3, lane 4), the pp6-specific signals in the range of 14 to 22 kDa were not detected because p6 had not been cleaved from the polyprotein. Importantly, however, no phosphorylated $Pr55^{Gag}$ protein was detected in this case either, and the same result was observed when cell lysates metabolically labeled in the presence of saquinavir were subjected to immunoprecipitation with antiserum against MA (Fig. 3, lane 5) or against CA (data not shown). These antisera efficiently precipitated ^{35}S-labeled $Pr55^{Gag}$ protein (data not shown), indicating that the Gag polyprotein was not phosphorylated. Accordingly, phosphorylation is likely to occur concomitant with or after release of p6, i.e., at the site of budding at the plasma membrane.

Phosphorylation of p6 by virion-associated kinase(s). If kinases phosphorylating p6 are present at the site of particle formation, they might also be incorporated into the budding virion. Furthermore, the presence of active cellular kinases within HIV-1 particles and their ability to modify MA or CA has been reported (6, 28). To determine whether highly purified HIV-1 particles contained kinases able to phosphorylate the endogenous p6 protein, unlabeled purified HIV-1 virions were gently lysed with Nonidet P-40. The lysate was subsequently incubated in the presence of divalent cations and [γ-^{32}P]ATP. As shown in Fig. 4A, phosphorylation of a number of virion-associated proteins was observed when incubation was performed in the presence of Mn^{2+}. Essentially the same pattern was obtained when purified HIV-1 particles were treated with subtilisin to digest kinases which may be peripherally associated with virus particles but are not present inside the virion (data not shown).

Immunoprecipitation of material obtained by in vitro phosphorylation revealed that MA and CA were weakly phosphorylated by virion-associated kinases while the main labeled product corresponded to pp6 (Fig. 4B). Similar to the results observed for pp6 immunoprecipitated from infected cells, the main αp6-precipitable band exhibited an apparent mobility of ca. 20 kDa on low-cross-linking Tris-glycine-SDS gels (Fig. 4B), while it migrated at ca. 8 kDa on Tris-Tricine gels (data not shown). This result suggests hyperphosphorylation of p6 in the in vitro reaction as well. However, comparison of the pattern of phosphoproteins labeled during the in vitro incubation with the material obtained by phosphorylation within infected cells also revealed significant differences in the pattern of pp6 isoforms (Fig. 4C). The main pp6 band obtained in vitro appears to correspond to one of the products observed after intracellular labeling, while several additional pp6 isoforms are detected in immunoprecipitations from infected cells.

Phosphorylation of viral proteins may be dependent on the particular host cell or on the viral isolate used. We therefore performed endogenous in vitro phosphorylation reactions using HIV-1_{NL4-3} particles purified from a different host cell line, as well as particles from a primary HIV-1 isolate. The patterns of phosphoproteins obtained were very similar in all cases, indicating that p6 is the major substrate of virion-associated

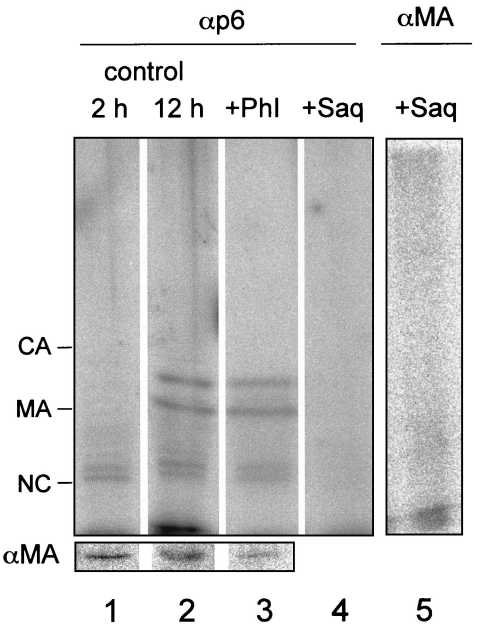

FIG. 3. Influence of phosphatase inhibitors or an HIV-1 PR inhibitor on p6 phosphorylation. HIV-infected MT-4 cells were metabolically labeled with [*ortho*-^{32}P]phosphate for 2 (lane 1) or 12 (lane 2) h. In parallel, infected cells were grown in the presence of a phosphatase inhibitor (PhI) cocktail (lane 3) or a protease inhibitor (saquinavir [Saq]; lanes 4 and 5), as described under Materials and Methods, and harvested 2 h after initiation of labeling. Cell extracts were subjected to immunoprecipitation using polyclonal antisera prepared against HIV p6 (lanes 1 to 4) or MA (lane 5 and small inserts below lanes 1 to 3), and immunoprecipitates were resolved by SDS-PAGE (17.5%; 200:1). The positions of major virion proteins as determined using ^{35}S-labeled virus are indicated at the left.

kinases (data not shown). There was also no difference in the pattern of labeled proteins when we compared the in vitro phosphorylation results observed for wild-type NL4-3 particles with those for an NL4-3 variant lacking Nef (Δ*nef* [59]) (data not shown).

To determine whether the virion-associated kinases also phosphorylate exogenously added substrates, purified recombinant HIV-encoded proteins were added to the in vitro kinase reaction. As shown in Fig. 5, a very strong signal was observed when GST-MA or GST-p6 was used, while GST alone or the viral proteins CA, RT, and IN were labeled only very weakly or not at all. Efficient in vitro phosphorylation was also observed for exogenously added His-tagged MA, which comigrated with endogenous pp6 (Fig. 5). These results indicate that p6 and MA are the major substrates for virion-associated kinases when added exogenously, while MA is only weakly phosphorylated in the endogenous reaction, and p6 is the main substrate in this case. In the case of exogenously added pGST-p6, the only product observed in a Coomassie blue-stained gel corresponded to the faster-migrating and less heavily phosphorylated form, while strongly labeled pGST-p6 with reduced electrophoretic mobility was also observed, again suggesting hyperphosphorylation of a minor fraction of p6-containing molecules.

Inhibition of p6 phosphorylation by kinase inhibitors. In order to address the question of which virion-associated kinase(s) may be responsible for p6 phosphorylation, we added specific kinase inhibitors to the in vitro phosphorylation reaction. In addition to some broad-range kinase inhibitors, we included olomoucine, an inhibitor of cyclin-dependent kinases (Cdks) and the related kinases ERK1 and ERK2 (58), since ERK2 has been reported to be incorporated into HIV-1 particles (6, 28). Inhibitors were added to a standard in vitro kinase reaction without exogenously added substrate, and the intensity of the labeled pp6 band was quantified by phosphorimage analysis (Fig. 6A). Among the inhibitors tested, only olomoucine resulted in consistent and significant reduction of pp6 intensity. Less pronounced inhibition was observed with genistein, a broad-range inhibitor of Tyr kinases, whereas the other inhibitors did not affect p6 phosphorylation in this assay. Consistent with the interpretation that several kinases are involved, phosphorylation could not be blocked completely by olomoucine but was reduced by approximately 40%. This result was confirmed using roscovitine, an inhibitor of the same group of kinases (34), which inhibited phosphorylation of p6 by virion-associated kinases in a concentration-dependent manner (Fig. 6B) with a maximal reduction by ca. 60%. Thus, we conclude that p6 can be phosphorylated by a kinase belonging to the Cdk/ERK group but that other kinases are also involved in the virion-associated kinase reaction. The amount of roscovitine required for inhibition suggests that ERK1 or ERK2 (50% inhibitory concentration, 34 or 14 μM, respectively [34]) rather than Cdks (50% inhibitory concentration, 200 to 700 nM) is affected. Since active ERK2 has already been detected in HIV-1 virions (6, 28), it appears likely that the effects of olomoucine and roscovitine seen here are due to the inhibition of virion-asociated ERK2. Furthermore, additional kinases not packaged in the virion appear to be involved in p6 phosphorylation in infected cells, and further studies are required to identify the relevant enzymes.

DISCUSSION

We have shown here that the HIV-1 late-domain protein p6 is phosphorylated at multiple sites in infected cells and virions and that pp6 constitutes the major phosphoprotein of HIV-1 particles. Similar to the ubiquitinylation of approximately 2% of virion-associated p6 described by Ott et al. (38), phosphorylation of p6 apparently involves only a small percentage of p6 molecules. Differential phosphorylation of a minor fraction of p6 at multiple sites can explain why pp6 has not been detected in biochemical analyses of purified HIV-1 (24). Interestingly, several other viral late-domain proteins have also been shown to be partially phosphorylated. For Moloney murine leukemia virus, a major phosphoprotein species detected in metabolically labeled virions is the multiply phosphorylated Gag protein pp12, which also carries the late-domain PPXY motif (49, 50, 66). In Mason-Pfizer monkey virus, the main phosphoprotein found is pp24 or its C-terminal cleavage product pp16 (3), which again harbor PPXY and P(T/S)AP motifs and have a function in virus release (64). The late-domain protein M is one of the two phosphoproteins of VSV (8). In all cases reported, phosphorylation involves only a fraction of the molecules and occurs at Ser or Thr residues, but in the case of VSV M, pTyr has also been detected (9). We suspect that late-domain protein phosphorylation may be a common feature

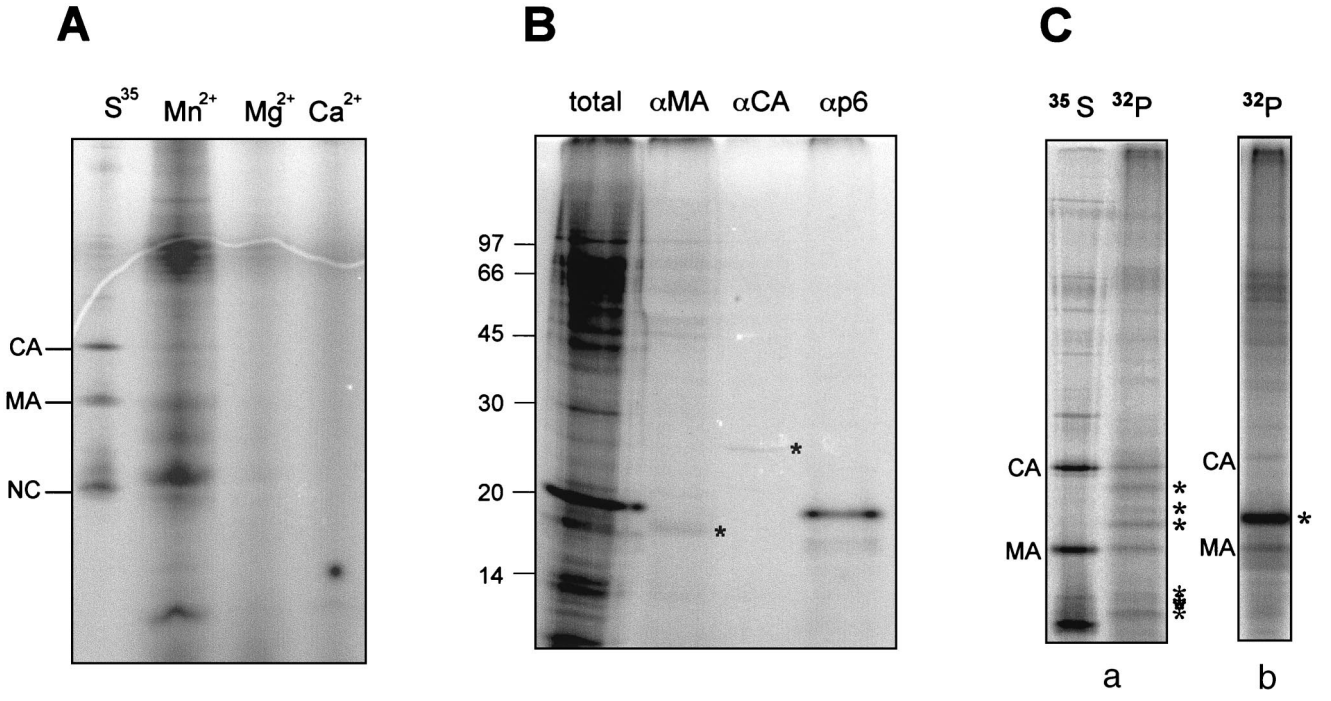

FIG. 4. Phosphorylation of HIV-1 proteins by virion-associated kinase(s). Unlabeled virus was purified and lysed, and in vitro phosphorylation was carried out as described under Materials and Methods. The reactions were stopped by the addition of 25 mM EDTA, the products were separated by SDS-PAGE, and labeled proteins were visualized by phosphorimage analysis. (A) The indicated divalent cations (5 mM) were added to the reaction mixture. The reaction products were separated on a Tris-Tricine-SDS gel (16.5%; 33:1). (B) Virus proteins were phosphorylated in vitro by virion-associated kinases in the presence of Mn^{2+} (lane total), and MA, CA, and p6 were immunoprecipitated from the reaction mixture using the indicated polyclonal antisera. The asterisks indicate the positions of pCA and pMA bands. The positions of marker proteins are indicated on the left, with their masses given in kilodaltons. (C) Comparison of phosphorylated virus-associated proteins from metabolically labeled cells (a) and after in vitro labeling (b), both separated on Tris-glycine-SDS gels (17.5%; 200:1). The asterisks indicate bands which can be immunoprecipitated by αp6 serum.

and might be revealed upon closer inspection in other retroviruses as well.

In the case of retroviruses, the functional relevance of phosphorylation sites in other domains of Gag has been investigated by mutational analyses. Substitution of the major phosphorylation site in Rous sarcoma virus MA has no effect on budding efficiency or virus infectivity (37), and mutation of each of three phosporylated serines in HIV-1 CA also does not impair virus release (7). However, phosphorylation mutants of p6 or the late-domain-bearing proteins of other viruses mentioned above have not been investigated in this respect. A functional relevance of phosphorylation is assumed in the case of the cellular membrane proteins ENaC and connexins, which bear late-domain-like sequences and undergo ubiquitin-mediated internalization. For both proteins, it was found that phosphorylation at multiple sites does occur, and it is presumed to regulate their turnover (31, 51). Although the mechanistic consequences of the known posttranslational modifications are not understood in either case, it can be speculated that the transmission of similar signals through posttranslational modification, or the attraction of enzymes that mediate posttranslational modification of a particular cellular protein at the membrane, is important in the case of both endocytosis and virus release.

Although p6 of NL4-3 comprises only 52 amino acids, 13 of these residues could potentially be phosphorylated, and 7 of these are predicted to be potential phosphorylation sites according to the NetPhos (1) algorithm. Our results show that phosphorylation in p6 does indeed occur at multiple residues and involves more than one cellular kinase. Further analysis by phosphopeptide mapping to determine the amino acid residues involved was precluded by the limited amount of metabolically labeled material obtainable after purification. Thus, extensive mutational analysis will be required to determine which of the 13 residues are modified and to dissect the functional relevance of the modifications. Since phosphoamino acid analysis indicated the presence of phosphotyrosine and there is only a single Tyr residue at position 36 of NL4-3 p6, modification at Y36 would be predicted to occur. Indeed, p6 was found to be specifically phosphorylated at Y36 in *E. coli* cells expressing Elk tyrosine kinase (B. Müller and H.-G. Kräusslich, unpublished observations). However, the fact that this residue is not highly conserved among HIV-1 isolates (being found in most subtype B sequences but less frequently in other subtypes) argues against a functional importance of Y36 phosphorylation. Moreover, published results from mutational analyses indicate that Y36 is not essential for p6 function. In a study of internal proteolytic cleavage within p6, Ott et al. exchanged Y36 for Ser, Cys, or Phe, respectively, and reported wild-type infectivity for the Y36S and Y36C variants (39). Thus, we believe that phosphorylation by Ser/Thr kinase(s) is a more likely candidate for a functionally relevant

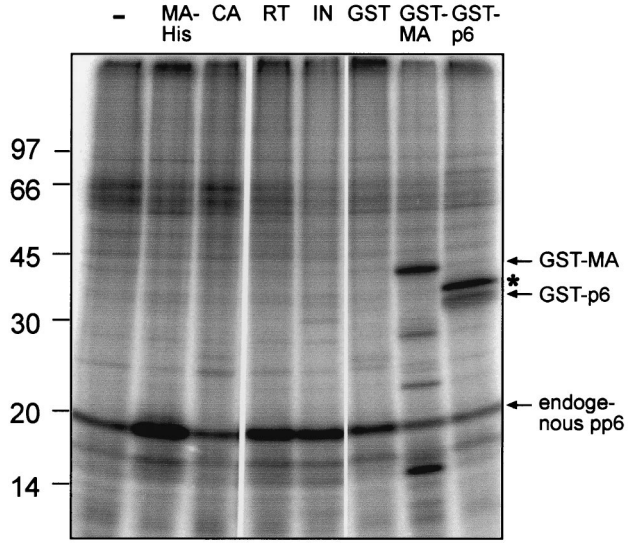

FIG. 5. Phosphorylation of exogenously added recombinant HIV-1 proteins by virion-associated kinases. Virion-associated kinase reactions were carried out as described under Materials and Methods. The indicated HIV-1-derived proteins (1.5 μg) purified from *E. coli* after recombinant expression were added to the reaction mixture (lane −, no addition). After 20 min at 30°C, the reactions were stopped by the addition of EDTA, and the proteins were separated by SDS-PAGE (17.5%; 200:1). Labeled proteins were visualized by phosphorimage analysis. The positions of marker proteins are indicated on the left, with their masses given in kilodaltons. The arrows at the right mark the positions of unlabeled GST-MA and GST-p6 as determined by Coomassie blue staining. An asterisk indicates a slower-migrating hyperphosphorylated form of GST-pp6.

modification. Experiments to identify target sites for Ser or Thr phosphorylation by the introduction of specific point mutations are under way.

p6 was also found to be the major labeled viral protein when phosphorylation was carried out by virion-associated kinases in vitro. Although others have previously carried out similar in vitro experiments using different strains of HIV-1, phosphorylation of p6 under these conditions has not been reported. This may reflect differences in the way the virus was grown and purified. In addition, pp6 might have gone unrecognized in previous studies due to its changed mobility on SDS-polyacrylamide gels. Depending on the gel system used, it can migrate very close to the viral MA protein. In fact, in their initial study of HIV particle-associated kinases, Cartier et al. observed two unidentified, strongly phosphorylated bands of virus-associated proteins migrating at positions corresponding to 15 and 17 kDa which could not be immunoprecipitated by antisera directed against MA (6).

If phosphorylation of p6 is indeed functionally important, the observed multiple phosphorylation of p6 could be either a functional requirement, a "bystander effect" caused by its function as a molecular adapter, or a mixture of both. A similar discussion has been ongoing in the case of p6 ubiquitinylation (see reference 57 for a review). A fraction of p6 itself is ubiquitinylated in the virion, but impairment of p6 ubiquitinylation by the exchange of its two Lys residues did not affect virus release, indicating that modification of p6 itself is not functionally essential (40). This may mean that ubiquitinylation can be shifted to other sites within Gag. Alternatively, a main function of p6 could be to recruit a ubiquitinylating activity to the site of assembly, thereby inducing the modification of a cellular protein required for budding. By analogy, p6 could serve as an adapter protein recruiting kinases to mediate phosphorylation of cellular budding factors. In this case, p6 phosphorylation may occur adventitiously due to its proximity to a kinase active site and its high content of serine and threonine residues. Equally possible, phosphorylation of p6 may be important for p6 function. The finding that only a small fraction of p6 mol-

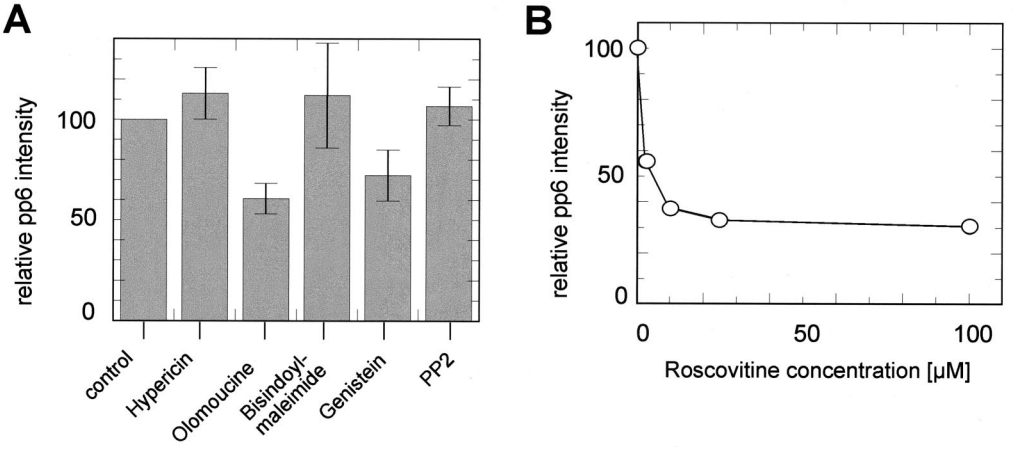

FIG. 6. Inhibition of virion-associated kinases by specific inhibitors. (A) The indicated inhibitors (Calbiochem) were added to an in vitro kinase reaction performed as described under Materials and Methods. The final concentrations of inhibitors were 10 μM hypericin, 100 μM olomoucine, 200 nM bisindoylmaleimide, 100 μM Genistein, and 50 nM PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo(3,4-α)pyrimidine]. Since inhibitor stock solutions were prepared in dimethyl sulfoxide (DMSO), the same final concentration (1%) of DMSO was added to the control reaction. The intensity of the endogenous pp6 band was quantitated by phosphorimaging, and values obtained in the presence of inhibitor were normalized to the value obtained in the control reaction carried out in parallel. The experiment was performed using lysates from NL4-3 or a primary HIV-1 isolate. Since these yielded very similar results, values were averaged from both. Mean values (± standard deviations) from three experiments are shown. (B) In vitro phosphorylation reactions were carried out using NL4-3 virus lysate in the presence of 1% DMSO or the indicated concentration of roscovitine (Calbiochem), respectively. The experiment was performed and evaluated as for panel A.

ecules was phosphorylated in this study does not contradict this hypothesis. First, phosphorylation and dephosphorylation are highly dynamic processes, and phosphorylation may occur only transiently at a specific point in the viral life cycle. Secondly, genetic-complementation experiments have indicated that a functional late domain is not required on all Gag molecules for budding to occur (61). One possibility would be that phosphorylation regulates ubiquitinylation as described for other proteins. More generally, a major function of late domains seems to be interaction with cellular factors. Phosphorylation of p6 may either generate or mask binding sites recognized by cellular proteins, thereby regulating late-domain function. Most likely, such regulation would be required at the plasma membrane around the time of virus budding; thus, the finding that p6 is phosphorylated in infected cells concomitant with or shortly after proteolytic cleavage of $Pr55^{Gag}$ would agree with this hypothesis. Of particular interest in this regard is the fact that phosphorylation can create specific binding sites for WW domains. Specifically, pSer-Pro or pThr-Pro motifs can be recognized by WW domain-carrying proteins (32). The generation of pThr-Pro within $p6_{NL4-3}$ is theoretically possible, since the protein contains a Thr-Pro motif, and Ser- or Thr-Pro also reflects the minimal consensus sequence for phosphorylation by ERK (see reference 13 for a review). Ongoing experiments in our laboratory address this possibility.

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