JOURNAL OF VIROLOGY, Feb. 2005, p. 2484–2492 Vol. 79, No. 4
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.4.2484–2492.2005

Phosphorylation and Proteolytic Cleavage of Gag Proteins in Budded Simian Immunodeficiency Virus

Sarah M. Rue,[1,2] Jason W. Roos,[1,2] Patrick M. Tarwater,[3] Janice E. Clements,[1,2] and Sheila A. Barber[1]*

Department of Comparative Medicine[1] and Department of Molecular Biology and Genetics,[2] Johns Hopkins University School of Medicine, Baltimore, Maryland, and School of Public Health, University of Texas Health Science Center at Houston, El Paso, Texas[3]

Received 19 March 2004/Accepted 5 October 2004

The lentiviral Gag polyprotein ($Pr55^{Gag}$) is cleaved by the viral protease during the late stages of the virus life cycle. Proteolytic cleavage of $Pr55^{Gag}$ is necessary for virion maturation, a structural rearrangement required for infectivity that occurs in budded virions. In this study, we investigate the relationship between phosphorylation of capsid (CA) domains in $Pr55^{Gag}$ and its cleavage intermediates and their cleavage by the viral protease in simian immunodeficiency virus (SIV). First, we demonstrate that phosphorylated forms of $Pr55^{Gag}$, several CA-containing cleavage intermediates of $Pr55^{Gag}$, and the free CA protein are detectable in SIV virions but not in virus-producing cells, indicating that phosphorylation of these CA-containing Gag proteins may require an environment that is unique to the virion. Second, we show that the CA domain of $Pr55^{Gag}$ can be phosphorylated in budded virus and that this phosphorylation does not require the presence of an active viral protease. Further, we provide evidence that CA domains (i.e., incompletely cleaved CA) are phosphorylated to a greater extent than free (completely cleaved) CA and that CA-containing Gag proteins can be cleaved by the viral protease in SIV virions. Finally, we demonstrate that $Pr55^{Gag}$ and several of its intermediates, but not free CA, are actively phosphorylated in budded virus. Taken together, these data indicate that, in SIV virions, phosphorylation of CA domains in $Pr55^{Gag}$ and several of its cleavage intermediates likely precedes the cleavage of these domains by the viral protease.

The *gag* gene of lentiviruses such as human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) encodes a polyprotein, $Pr55^{Gag}$, which is composed of six distinct domains (from N terminus to C terminus): matrix (MA), capsid (CA), p2, nucleocapsid (NC), p1, and p6 (22, 24, 37, 39). In approximately 5% of *gag* translation events, a ribosomal frameshift causes the production of the $Pr160^{Gag\text{-}Pol}$ polyprotein, which contains the viral enzymes protease (PR), reverse transcriptase, and integrase at the C terminus of the $Pr55^{Gag}$ polyprotein (27). Because $Pr55^{Gag}$ and $Pr160^{Gag\text{-}Pol}$ are incorporated into the virion at approximately the same levels at which they are translated, the virion is composed mostly of $Pr55^{Gag}$. At an undetermined point in the late stages of the lentivirus life cycle, PR cleaves itself out of the $Pr160^{Gag\text{-}Pol}$ polyprotein, homodimerizes, and separates the constituent domains of $Pr55^{Gag}$, eventually releasing each of these domains as free proteins that have independent functions (for a review, see reference 11). Because the rate of cleavage (also referred to as proteolytic processing) by PR is different at each of the cleavage sites in $Pr55^{Gag}$, several cleavage intermediates are detectable both in virions and in virus-producing cells (10, 38, 39).

The proteolytic processing of $Pr55^{Gag}$ enables the virion to undergo the process of maturation, a structural rearrangement in which MA stays primarily associated with the virion envelope while CA forms a cone-shaped core around NC and the viral RNA (16; for reviews, see references 15 and 17). Virion maturation and the prerequisite $Pr55^{Gag}$ cleavage are absolutely required for virion infectivity (32, 36, 44). It is generally agreed that virion maturation occurs outside the cell in lentiviruses, since budding virions have an immature morphology and mature virions are visible only outside the cell in electron micrographs of most virus-producing cells (macrophages are one exception) (17, 25, 33, 42). The timing of proteolytic cleavage of the $Pr55^{Gag}$ polyprotein is less clear, however, as $Pr55^{Gag}$, its cleavage intermediates, and its final cleavage products (free MA, CA, etc.) are all present both in budded virions and in virus-producing cells (39). In HIV-1, it has been shown that $Pr55^{Gag}$ cleavage begins at the plasma membrane of the cell in the budding virion (30, 31), but a consensus has not been reached on whether cleavage is completed inside the budding virion before it pinches off from the plasma membrane or inside the budded virion that has been released from the cell. Furthermore, very little is known about proteolysis of $Pr55^{Gag}$ in SIV.

The processes of $Pr55^{Gag}$ cleavage and virion maturation are believed to be highly regulated (13, 14, 45, 46, 53), although the mechanisms of this regulation are not entirely clear. Some groups have proposed that phosphorylation of retroviral Gag proteins enhances their cleavage by PR (41, 55). HIV-1 studies have demonstrated that the free (completely cleaved) CA protein is posttranslationally modified by phosphorylation (7, 9, 10, 38, 39), and some groups have observed phosphorylated $Pr55^{Gag}$ (6, 10).

We became interested in the relationship between lentiviral CA phosphorylation and proteolytic processing of $Pr55^{Gag}$

* Corresponding author. Mailing address: 733 N. Broadway Rm. 831, Baltimore, MD 21205. Phone: (410) 955-9770. Fax: (410) 955-9823. E-mail: sabarber@jhmi.edu.

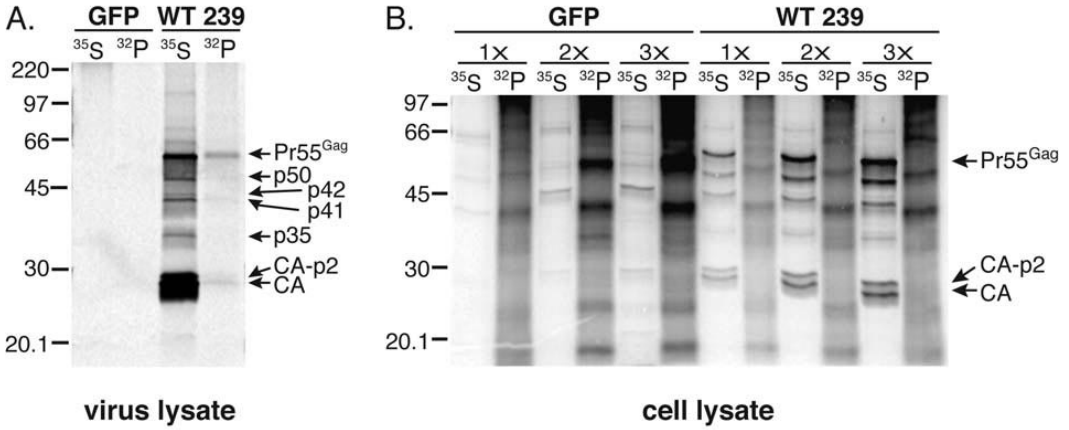

FIG. 1. Analysis of SIV Gag phosphorylation in virions and virus-producing cells. (A) Virus lysates derived from 293T cells transfected with a GFP control vector or wild-type (WT) SIVmac239 proviral DNA and labeled with [^{35}S]Met/Cys (^{35}S) or [^{32}P]orthophosphate (^{32}P) were immunoprecipitated with IgG-purified SIV capsid (CA) polyclonal antiserum, resolved by SDS-PAGE, and visualized by phosphorimager analysis. (B) Cell lysate protein from the transfection in panel A (500 μg [1×], 1 mg [2×], or 1.5 mg [3×]) was immunoprecipitated with IgG-purified SIV CA antiserum as described in Materials and Methods and resolved by SDS-PAGE.

when we detected phosphorylated forms of the free CA protein and several polyproteins that contain CA as a domain ($Pr55^{Gag}$ and some of its cleavage intermediates) in SIV virions but not in virus-producing cells (our unpublished observations). Here, we investigate this relationship in detail. By analyzing ^{35}S- or ^{32}P-labeled virus produced from cells transfected with a protease-inactive mutant of SIVmac239, we demonstrate that phosphorylation of SIV $Pr55^{Gag}$ can occur and does not require the presence of a functional viral protease. By cleaving $Pr55^{Gag}$ in ^{35}S- and ^{32}P-labeled wild-type and protease mutant virus with exogenous protease, we show that the CA domain of $Pr55^{Gag}$ can be phosphorylated in SIV virions. Furthermore, we provide evidence that the CA domain of $Pr55^{Gag}$ and/or its cleavage intermediates is phosphorylated to a greater extent than free, completely cleaved CA protein. By assaying changes in Gag protein levels over time in virus incubated in vitro, we demonstrate that SIV PR can cleave Gag proteins in budded virions. Using a similar assay, we show that the phosphorylation of $Pr55^{Gag}$ and CA-containing cleavage intermediates, but not the phosphorylation of free CA protein, increases over time in the virion, which indicates that phosphorylated free CA is likely derived from cleavage of phosphorylated CA domains by PR. Taken together, these results strongly suggest that, in budded SIV virions, phosphorylation of CA domains occurs before they are completely cleaved by PR.

MATERIALS AND METHODS

Plasmid construction. The previously characterized clones of the 5′ (p239SpSp5′) and 3′ (p239SpE3′) halves of the SIVmac239 genome (AIDS Research and Reference Reagent Program) (34, 47) were used to generate the full-length $D(25)A_{PR}$ SIVmac239 mutant exactly as described previously (49). The primers used to create this viral mutant made a (GAT→GCT) nucleotide substitution in codon 25 of the protease-encoding portion of *pol*. The full-length clone $D(25)A_{PR}$ SIVmac239 pBS$^-$ was verified by complete sequencing of the viral coding region.

Cell culture. Human embryonic kidney 293T cells (American Type Culture Collection, Manassas, Va.) were maintained as described previously (3), except that 0.5 mg of gentamicin/ml was used instead of penicillin-streptomycin.

Metabolic labeling and immunoprecipitation of Gag proteins. 293T cells at ~40% confluence in 10-cm-diameter dishes or 150-cm^2 flasks were transfected with a green fluorescent protein (GFP) control plasmid (pEGFP-N1; BD Biosciences Clontech, Palo Alto, Calif.) or infectious viral DNA with Lipofectamine and Plus reagents (Invitrogen Corporation, Carlsbad, Calif.) according to the manufacturer's recommendations. The next day, cells were metabolically labeled with [^{35}S]Met/Cys alone or [^{35}S]Met/Cys and [^{32}P]orthophosphate in parallel. For ^{35}S labeling, culture medium was replaced with methionine- and cystine-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 2% fetal bovine serum (Atlanta Biologicals, Norcross, Ga.), 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, and 0.5 mg of gentamicin/ml (mcfDMEM), and cells were serum starved for 30 min at 37°C. The medium was then replaced with fresh mcfDMEM containing ~0.1 mCi of Tran[^{35}S]-Label ([^{35}S]Met/Cys; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.)/ml, and cells were incubated for 1 to 2 h at 37°C. For ^{32}P labeling, culture medium was replaced with phosphate-free DMEM (Invitrogen) supplemented with 5% dialyzed fetal bovine serum (Invitrogen), 2 mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, and 0.5 mg of gentamicin/ml, and cells were incubated for 30 min at 37°C. The medium was replaced with fresh phosphate-free DMEM containing [^{32}P]orthophosphate (Perkin-Elmer Life and Analytical Sciences, Inc., Boston, Mass.) at ~1.0 mCi/ml, and cells were incubated for 2 h at 37°C. In experiments in which cells were labeled in parallel with ^{35}S and ^{32}P, [^{35}S]Met/Cys or [^{32}P]orthophosphate was added to parallel cultures simultaneously, and both cultures were labeled for 2 h.

Cell supernatants containing radiolabeled virus were pelleted briefly and filtered through a 0.45-μm-pore-size syringe filter (Millipore, Bedford, Mass.) to remove cell debris. Radiolabeled virus was purified by centrifugation at 133,000 × *g* for 2 h at 4°C through a cushion of 20% sucrose in TNE buffer (20 mM Tris [pH 8.0], 150 mM NaCl, and 2 mM EDTA). Virus pellets were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (phosphate-buffered saline with 1% [vol/vol] Igepal [NP-40], 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM sodium orthovanadate, and protease inhibitor cocktail III [Calbiochem, San Diego, Calif.]). Radiolabeled cells were washed once with ice-cold phosphate-buffered saline and lysed in RIPA buffer, and cell lysates were clarified by sonication and centrifugation.

For immunoprecipitation reactions, cell (500 μg of protein) and virus lysates prepared as described above were incubated for at least 1 h at 4°C with 3 μg of immunoglobulin G (IgG)-purified rabbit SIV CA polyclonal antiserum (HRP, Denver, Pa.). Protein A agarose beads (Sigma-Aldrich, St. Louis, Mo.) were then added, and lysates were incubated overnight at 4°C. Immunoprecipitates were washed with RIPA buffer and separated on 12.5% Tris-HCl Criterion precast gels (Bio-Rad, Hercules, Calif.) that were fixed and subjected to phosphorimager analysis with a Typhoon 9210 phosphorimager and Molecular Dynamics ImageQuant version 5.2 software (Amersham Pharmacia, Piscataway, N.J.). In experiments in which Gag was immunoprecipitated from 500 μg, 1 mg, and 1.5 mg of cell lysate protein (Fig. 1B), 3, 6, and 9 μg of IgG-purified SIV CA antiserum

were used for immunoprecipitation, respectively, and volumes of the immunoprecipitation reactions were scaled up accordingly.

HIV-2 protease cleavage assay. 293T cells were transfected and metabolically labeled as described above, with the exception that cells were labeled in parallel with 0.04 mCi of [^{35}S]Met/Cys/ml and 0.8 mCi of [^{32}P]orthophosphate/ml for 2 h. Labeled virus-containing cell supernatants were pelleted briefly and filtered, and virus was purified by centrifugation as described above. Purified virus was then lysed, divided in half, and incubated with either HIV-2 protease (PR) (AIDS Research and Reference Reagent Program) (48) or PR suspension buffer (100 mM $NaPO_4$ [pH 8.0] and 50 mM NaCl) in cleavage buffer (10 mM Tris-HCl [pH 7.0], 130 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) for 15 h at 37°C. Cleavage products were directly resolved on a 15% Tris-HCl Criterion precast gel (Bio-Rad), which was fixed and subjected to phosphorimager analysis and band densitometry. Ratios of ^{32}P to ^{35}S (^{32}P/^{35}S ratios) for Gag proteins (see Fig. 3 and 5) were determined by dividing the band intensity of the ^{32}P-labeled protein by the band intensity of the ^{35}S-labeled protein corrected for methionine and cysteine content. The ^{32}P/^{35}S ratio is a measure of the extent of protein phosphorylation and is unaffected by the actual amount of protein present in each sample, because the amount of protein phosphorylation (^{32}P band intensity) is standardized according to the amount of protein that is present in the sample (the ^{35}S band intensity).

Analysis of Gag protein cleavage and phosphorylation over time in budded virus. Virus derived from transfected 293T cells that had been labeled with ~0.2 mCi of [^{35}S]Met/Cys/ml or ~0.6 mCi of [^{32}P]orthophosphate/ml for 1 (^{35}S only) or 2 (^{35}S and ^{32}P in parallel) h as described above was briefly pelleted, filtered, aliquoted, and incubated at 37°C for the indicated amounts of time. At each time point, an aliquot was immediately put on ice and then placed at −80°C to stop further proteolytic activity. After the time course was complete, all aliquots were thawed in an ice bath, and virus was purified by centrifugation through 20% sucrose in TNE buffer, lysed, and immunoprecipitated at 4°C as described above. Immunoprecipitates were resolved on 12.5% Tris-HCl Criterion gels (Bio-Rad) that were fixed and subjected to phosphorimager analysis and band densitometry. Data points for the graph of the fraction of initial protein remaining over time (see Fig. 4C) were calculated from the band densitometry data as the band intensity of the protein at time point x ($t = x$) divided by the band intensity of the protein at the 0-min time point ($t = 0$). Data points for graphs of the phosphorylation (n-fold) of proteins over time relative to $t = 0$ (see Fig. 5C) were calculated by dividing the ^{32}P/^{35}S ratio (see above) for the protein at $t = x$ by the ^{32}P/^{35}S ratio for the protein at $t = 0$.

RESULTS

Phosphorylated capsid-containing Gag proteins are detectable in SIV virions but not in cells. In our studies of the role of phosphorylation of SIV proteins in viral replication, we consistently observed that free CA protein, the SIV Pr55^{Gag} precursor polyprotein, and several CA-containing cleavage intermediates were phosphorylated in budded virions (our unpublished observations; see below). To examine whether these Gag proteins are also phosphorylated in SIV-producing cells, 293T cells transfected with a GFP control vector or infectious wild-type SIVmac239 DNA were in vivo labeled with [^{35}S]Met/Cys and [^{32}P]orthophosphate in parallel. Cell and virus lysates were immunoprecipitated with SIV CA polyclonal antiserum (immunoprecipitation is necessary for visualization of Gag proteins in cell lysates), resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and subjected to phosphorimager analysis (Fig. 1). By convention, in this and other assays presented in this paper, Gag protein that is detected in virus lysates represents Gag present in virus that has budded from the cell, while Gag protein that is detected in cell lysates represents both Gag present in the cytoplasm of the cell and Gag present in virions that are budding (i.e., not yet released) from the plasma membrane at the time of cell harvest.

Several CA-containing proteins were detected in ^{35}S-labeled wild-type SIV virus lysates: the Pr55^{Gag} precursor polyprotein, free CA protein, and five processing intermediates that we have named according to their apparent molecular masses determined from this and other experiments (p50, p42, p41, p35, and p28 [CA-p2]) (Fig. 1A). The CA-p2 protein is the last processing intermediate in the cleavage of lentiviral CA, such that cleavage of the CA-p2 boundary by PR produces free CA (39, 45, 46, 52). For the sake of simplicity, from this point on we refer to CA that has been completely cleaved as "free" and CA that is present as a domain in Pr55^{Gag} and/or the cleavage intermediates as "domain form(s) of CA" or "CA domains."

Pr55^{Gag}, p50, p41, CA-p2, and a 27-kDa protein that was confirmed by mass spectroscopy to be SIV CA (data not shown) were all detectably phosphorylated in ^{32}P-labeled virus (Fig. 1A), indicating that most but not all of the different cleavage forms of the CA protein are phosphorylated in the virion. To our knowledge, phosphorylated Pr55^{Gag} has been observed in only one other study in a system using full-length HIV or SIV virus: it was detected intracellularly in a T-lymphocyte line infected with HIV-1 (10). Furthermore, the detection of phosphorylated forms of cleavage intermediates of Pr55^{Gag} in lentiviral virions has not been reported. It is important to note that because the SIVmac239 p2 protein does not contain any of the amino acids that are typical acceptors for phosphate modification (serine, threonine, tyrosine, or histidine) (21, 51), the CA domain, not the p2 domain, of the CA-p2 intermediate is phosphorylated in SIV virions.

In contrast, no phosphorylated Pr55^{Gag}, cleavage intermediates, or free CA was detected in cell lysates, even in immunoprecipitates from 1.5 mg of cell lysate (three times the amount of lysate required for optimal detection of ^{35}S-labeled Gag proteins) (Fig. 1B). The same CA-containing Gag proteins were detected as phosphorylated proteins in budded virus but not in virus-producing cells when either primary rhesus macrophages or CEM×174 cells were used in similar assays (data not shown), indicating that the SIV Gag protein phosphorylation profile is independent of cell type. The lack of detectable phosphorylation of free SIV CA inside virus-producing cells presents a potential difference between SIV and HIV-1, since at least one study has shown convincingly that free HIV-1 CA is phosphorylated both inside the cell and in virus particles (40). Collectively, these data indicate that phosphorylation of CA-containing Gag proteins occurs predominantly (if not exclusively) inside the virion subsequent to budding and is likely mediated by one or more virion-associated kinases.

Phosphorylation of Pr55^{Gag} in a protease-inactive mutant of SIVmac239. In addition to CA, at least two other previously characterized phosphoproteins (MA and p6) (5, 12, 20, 39, 40) are also present as domains within lentiviral Pr55^{Gag}. To determine whether the phosphorylated Pr55^{Gag} polyprotein present in SIV virions was phosphorylated on CA or another domain, we initially wanted to cleave phosphorylated Pr55^{Gag} in virus lysates with exogenous protease and to analyze which of the resultant Pr55^{Gag} constituent proteins are phosphorylated. The limitation of using wild-type virus lysates in such an experiment is that they contain not only Pr55^{Gag} but also several of its CA-containing cleavage intermediates (Fig. 1A). The origin of free CA produced by treating wild-type virus lysates with exogenous protease would therefore be uncertain. To circumvent this problem, we constructed a viral mutant that encoded an alanine substitution at the active site aspartate of

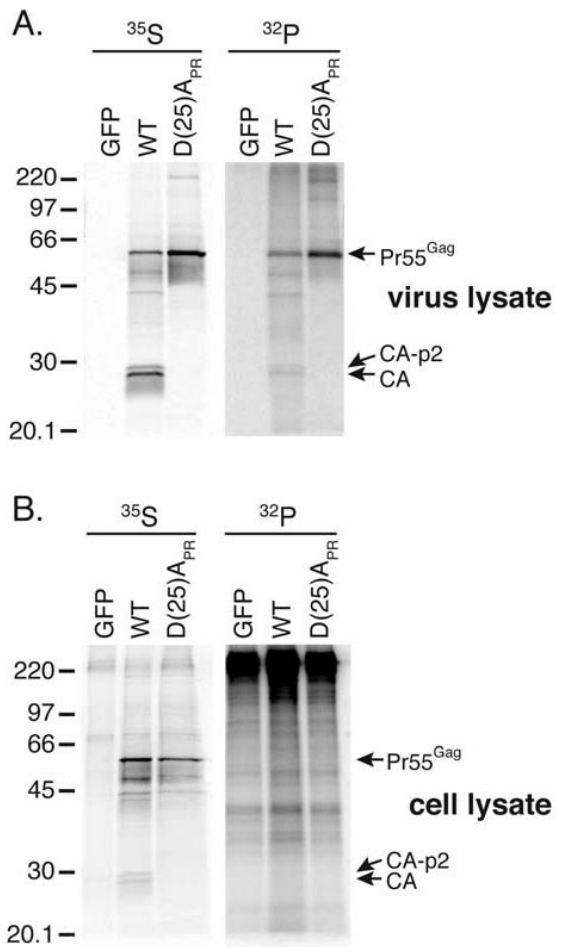

FIG. 2. Gag phosphorylation in a protease-inactive mutant of SIVmac239. Virus (A) and cell (B) lysates derived from ^{35}S- or ^{32}P-labeled 293T cells transfected with a GFP control vector, wild-type (WT) SIVmac239 proviral DNA, or D(25)A_{PR} SIVmac239 proviral DNA were immunoprecipitated with IgG-purified SIV CA antiserum, resolved by SDS-PAGE, and visualized with a phosphorimager.

PR, D(25)A_{PR} SIVmac239. Cells transfected with this mutant should produce virions that contain $Pr55^{Gag}$ but none of its processing intermediates, making it easier to examine the phosphorylation state of the CA domain of $Pr55^{Gag}$. We observed no detectable PR activity, virion maturation, or virus replication in our D(25)A_{PR} SIVmac239 mutant (data not shown), a phenotype identical to that reported for HIV-1 proviral clones with active site substitutions in PR (18, 36).

To determine whether $Pr55^{Gag}$ was phosphorylated in D(25)A_{PR} SIVmac239 virus, 293T cells transfected with a GFP control vector, wild-type viral DNA, or D(25)A_{PR} viral DNA were labeled in parallel with [^{35}S]Met/Cys and [^{32}P]orthophosphate, and virus and cell lysates from these samples were immunoprecipitated with IgG-purified SIV CA antiserum and resolved by SDS-PAGE (Fig. 2). As in wild-type virus lysates, $Pr55^{Gag}$ in D(25)A_{PR} virus lysates was phosphorylated, indicating that phosphorylation of $Pr55^{Gag}$ can occur in the absence of an active viral protease (Fig. 2A). Consistent with our earlier results, no intracellular phosphorylated Gag proteins were detected for either wild-type or D(25)A_{PR} SIVmac239 (Fig. 2B).

The CA and MA domains of $Pr55^{Gag}$ are phosphorylated in SIV virions. To examine whether the CA domain of $Pr55^{Gag}$ is phosphorylated in budded virus, we labeled 293T cells transfected with a GFP control vector, D(25)A_{PR} proviral DNA, or wild-type proviral DNA in parallel with ^{35}S or ^{32}P. Virus was purified by centrifugation, lysed, divided in half, and either mock treated or treated with exogenous HIV-2 PR (purified SIV PR is not readily available). The products of these reactions were then directly resolved by SDS-PAGE (Fig. 3). Phosphorylated forms of both free MA and free CA were detected in D(25)A_{PR} virus lysates that had been treated with HIV-2 PR, indicating that the phosphorylated $Pr55^{Gag}$ observed in wild-type virus is likely phosphorylated on both the MA and CA domains. Because we had difficulty detecting ^{35}S-labeled p6 in this and other assays (data not shown), we cannot determine whether the p6 domain of SIV $Pr55^{Gag}$ is phosphorylated as well.

Although, consistent with earlier results, phosphorylated

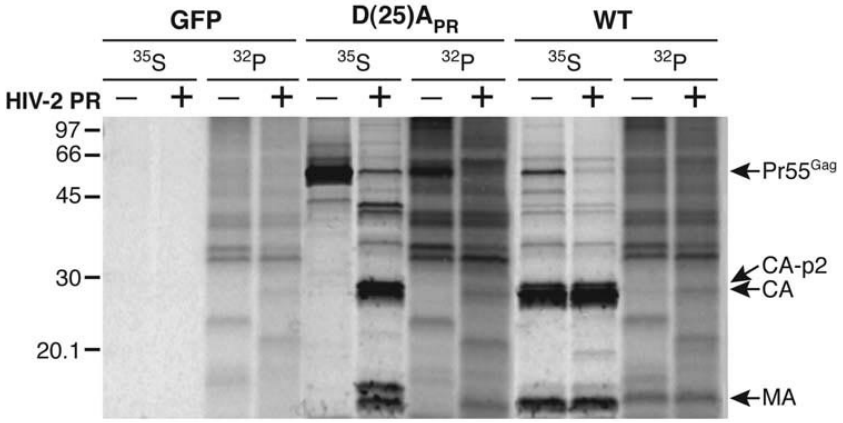

FIG. 3. Analysis of phosphorylation of the domain form of SIV CA. Virus lysates derived from ^{35}S- or ^{32}P-labeled 293T cells transfected with a GFP control vector, D(25)A_{PR} SIVmac239 proviral DNA, or wild-type (WT) SIVmac239 proviral DNA were divided in half and either treated with HIV-2 PR (+) or mock treated (−), and products were subjected to SDS-PAGE and phosphorimager analysis. Gel lanes were rearranged with Adobe Photoshop to accommodate the flow of the text. MA, free matrix protein.

free CA was detected in mock-treated wild-type virus lysates in this assay, there appeared to be more phosphorylated free CA in HIV-2 PR-treated wild-type virus lysates (Fig. 3). To quantitatively address this possibility, band densitometry was performed on the ^{35}S- and ^{32}P-labeled free CA protein in both mock-treated and HIV-2 PR-treated wild-type virus lysates. The band densitometry data were used to calculate the $^{32}P/^{35}S$ ratio for free CA in both mock-treated [$(^{32}P/^{35}S)_{mock}$] and HIV-2 PR-treated [$(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$] wild-type virus lysates as described in Materials and Methods. The free CA present in mock-treated lysates represents only CA that was completely cleaved by the innate (SIV) PR, while the free CA in HIV-2 PR-treated lysates represents both CA that was cleaved by the innate PR and CA that originated in the domain form in $Pr55^{Gag}$ and/or its cleavage intermediates but was released by treatment with exogenous (HIV-2) PR. Therefore, $(^{32}P/^{35}S)_{mock}$ represents the extent of phosphorylation of free CA, while $(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$ represents the extent of phosphorylation of both free CA and domain form CA. As such, if domain form CA is phosphorylated to the same extent as free CA, we would expect that $(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$ would equal $(^{32}P/^{35}S)_{mock}$. However, if domain form CA is phosphorylated to a greater extent than free CA, we would expect that $(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$ would be greater than $(^{32}P/^{35}S)_{mock}$.

Analysis of the band densitometry data for free CA in wild-type virus indicated that $(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$ was greater than $(^{32}P/^{35}S)_{mock}$: in this experiment, $(^{32}P/^{35}S)_{HIV\text{-}2\ PR}$ was 5.9, while $(^{32}P/^{35}S)_{mock}$ was 3.3. This trend was observed in three additional independent replicates of this experiment (data not shown). Therefore, the domain form of CA is phosphorylated to a greater extent than free (completely cleaved) CA protein in budded SIV virions. We cannot determine whether the phosphorylated free CA protein released by HIV-2 PR treatment of wild-type virus originated as a domain in $Pr55^{Gag}$ or in one of the CA-containing cleavage intermediates. However, it is very likely that at least some of this phosphorylated free CA originated as a domain in $Pr55^{Gag}$, since phosphorylated free CA was released from $Pr55^{Gag}$ when $D(25)A_{PR}$ SIVmac239 virus was treated with HIV-2 PR.

$Pr55^{Gag}$ processing in virions. We next identified the origin of phosphorylated free CA present in budded SIV virions. Phosphorylated forms of CA are detectable only in budded virus; thus, there are only two possible origins for phosphorylated free CA: either (i) phosphorylated domain forms of CA are cleaved within the virion to produce phosphorylated free CA or (ii) the domain forms of CA and free CA protein are independently phosphorylated by one or more virion-associated kinases.

As discussed above, no studies have directly addressed whether PR can cleave $Pr55^{Gag}$ and its intermediates in SIV virions. Therefore, to assess whether the first scenario is even possible, we assayed the proteolysis of CA-containing Gag proteins over time in virus-containing cell supernatants. 293T cells transfected with wild-type or $D(25)A_{PR}$ SIVmac239 were labeled with [^{35}S]Met/Cys, and virus-containing cell supernatants were briefly pelleted and filtered to remove cell debris. Filtered virus was then aliquoted and incubated at 37°C for different amounts of time. At each time point, an aliquot of virus was immediately frozen to stop proteolytic processing. When the time course was complete, all aliquots were thawed in an ice bath, virus was purified by centrifugation and lysed, and proteins were immunoprecipitated with IgG-purified SIV CA antiserum and resolved by SDS-PAGE (Fig. 4).

We observed that SIV $Pr55^{Gag}$ and all of the previously detected CA-containing cleavage intermediates (p50, p42, p41, p35, and CA-p2) are in fact cleaved over time in budded wild-type virus (Fig. 4A). Further, this cleavage is specific to the viral protease, since we observed very little change in $Pr55^{Gag}$ levels over time in protease-inactive $D(25)A_{PR}$ SIVmac239 virus (Fig. 4B). Band densitometry was performed for each protein, and the fraction of initial protein remaining over time was calculated for $Pr55^{Gag}$, free CA, and the cleavage intermediates (see Materials and Methods). All of the detectable cleavage of CA-containing Gag proteins in wild-type virus in this assay occurred between the 0-min and 6-h (360-min) time points (Fig. 4C).

Similar results were obtained for this time course experiment when [^{35}S]Met/Cys-labeled, 293T-derived lysed wild-type virus was analyzed by direct electrophoresis rather than immunoprecipitation (data not shown), indicating that the results of this experiment are not biased by immunoprecipitation. Moreover, similar trends were also observed when these time course experiments were performed with virus derived from transfection of COS-1 cells (data not shown), indicating that the intravirion cleavage of CA-containing Gag proteins by PR is not specific to 293T-derived virus.

It is clear that cleavage of SIV $Pr55^{Gag}$ and its intermediates does not occur exclusively in budded virions. Both cleavage intermediates of $Pr55^{Gag}$ and completely cleaved proteins such as free CA are present in budded virus produced from ^{35}S-labeled cells at the earliest time point at which it can be detected (these cleavage products are likely the result of proteolysis that occurs at the plasma membrane in the budding virion [our unpublished observations]). Furthermore, cleavage intermediates of $Pr55^{Gag}$ and free CA can be detected in lysates of ^{35}S-labeled cells producing SIV (Fig. 1B) (these cleavage products [like those in budded virus] are detected only when an active viral protease is present, and they likely originate from cleavage of $Pr55^{Gag}$ by PR both in the cytoplasm and in virus that is budding from the plasma membrane of the cell [30, 31]). Nevertheless, the results of our time course assays clearly indicate that SIV PR can cleave $Pr55^{Gag}$ and its cleavage intermediates in virus that has budded from the cell.

Phosphorylation of CA-containing Gag proteins over time in budded virus. Since cleavage of $Pr55^{Gag}$ by PR can occur in budded virus, it is possible that phosphorylated free CA protein is derived from the proteolytic release of phosphorylated domain forms of CA from $Pr55^{Gag}$ and/or its cleavage intermediates. However, we have not yet eliminated the possibility that free CA and proteins that contain CA as a domain ($Pr55^{Gag}$ and the phosphorylated cleavage intermediates p50, p41, and CA-p2) are independently phosphorylated. To examine this possibility, we labeled 293T cells transfected with wild-type SIVmac239 with ^{35}S (Fig. 5A) and ^{32}P (Fig. 5B) in parallel and performed the same assay shown in Fig. 4 with the budded virus. Consistent with earlier results, $Pr55^{Gag}$, free CA, and the CA-containing $Pr55^{Gag}$ cleavage intermediates p50, p41, and CA-p2 were detectably phosphorylated in budded virus (Fig. 5B). Band densitometry of these gels was performed, and the phosphorylation of each of the proteins relative to the 0-min

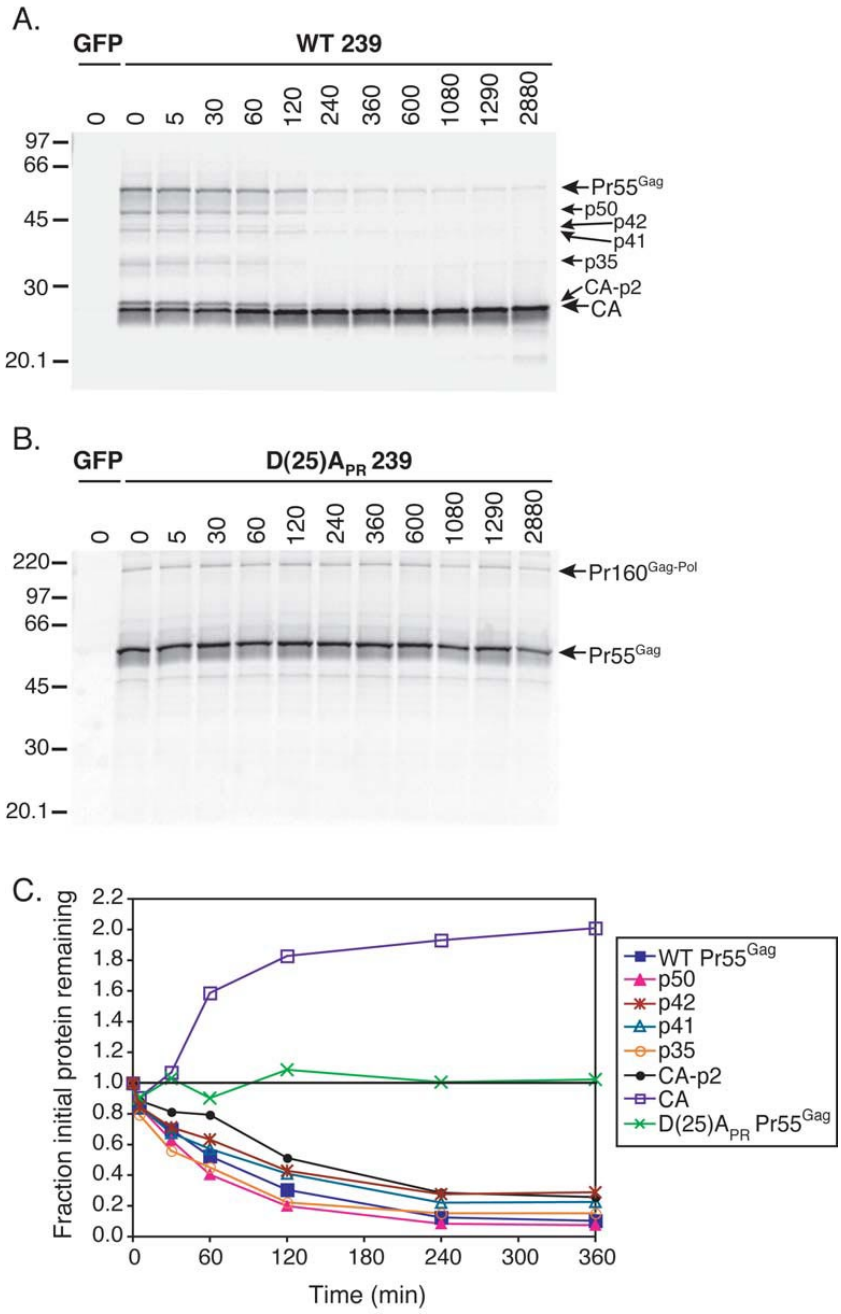

FIG. 4. Proteolytic processing of Gag in budded SIV virions. Virus-containing cell supernatants from 293T cells transfected with wild-type (A) or D(25)A_{PR} (B) proviral DNA and labeled with ^{35}S were aliquoted and incubated at 37°C for the indicated number of minutes. Upon completion of the time course, virus from each time point was purified, lysed, immunoprecipitated with SIV CA antiserum, resolved by SDS-PAGE, and visualized with a phosphorimager. (C) Fraction of initial protein remaining over time calculated for Gag proteins as described in Materials and Methods from band densitometry of the gels in panels A and B. Data for the first 6 h of the time course are shown, as cleavage of Gag proteins was not detected past the 6-h (360-min) time point. Results shown are representative of at least three independent experiments.

time point ($t = 0$) was calculated for each time point (Fig. 5C) (see Materials and Methods for calculations). We repeatedly observed that phosphorylation of $Pr55^{Gag}$ and, to a greater extent, phosphorylation of the cleavage intermediates p50, p41, and CA-p2 increased over time in budded virus (Fig. 5C, top panel). Although active phosphorylation of $Pr55^{Gag}$ and the cleavage intermediates p50, p41, and CA-p2 was detected throughout the entire 48-h time course, the greatest increases in phosphorylation of these proteins occurred between the 0-min and 6-h time points. In contrast, free CA phosphorylation did not significantly increase relative to the phosphorylation of the cleavage intermediates (Fig. 5C, bottom panel). Thus, we conclude that free CA is not likely to be actively phosphorylated over time by a virion-associated kinase. Fur-

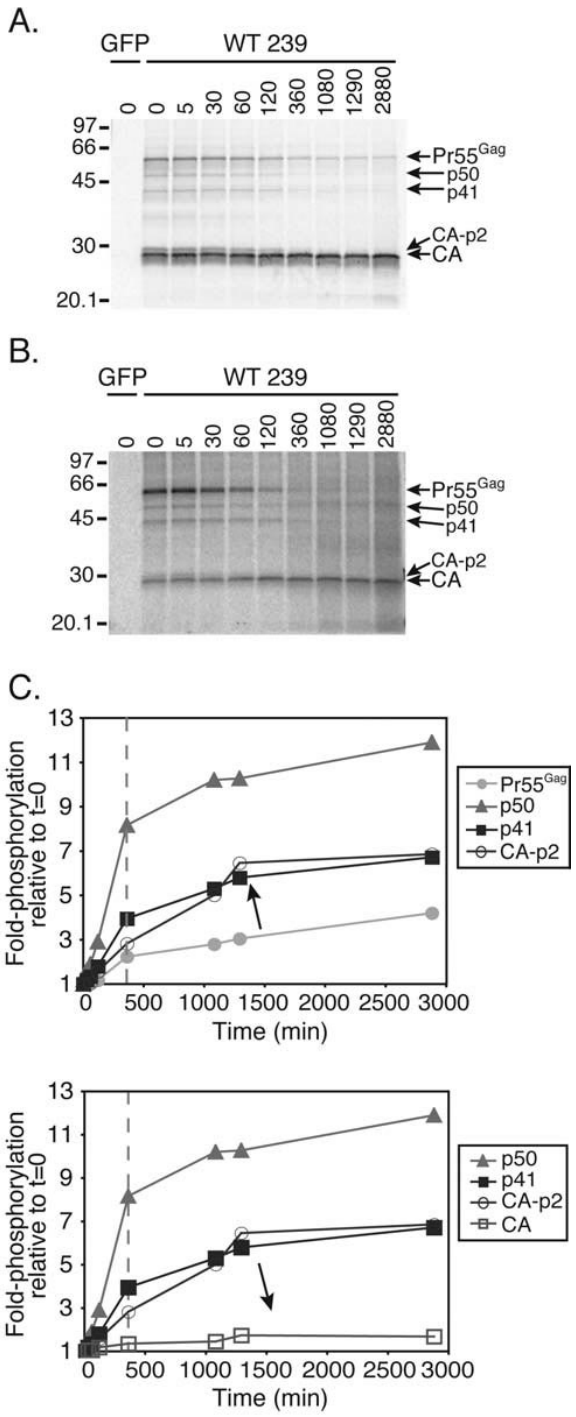

FIG. 5. Phosphorylation of Gag proteins over time in budded virus. ^{35}S-labeled (A) or ^{32}P-labeled (B) 293T-derived wild-type SIVmac239 virus-containing cell supernatants were assayed as described in the legend to Fig. 4. (C) Phosphorylation (*n*-fold) of Gag proteins over time relative to the 0-min time point ($t = 0$), calculated from band densitometry data from the gels in panels A and B as described in Materials and Methods. Top panel, Pr55^{Gag} versus cleavage intermediates; bottom panel, free CA versus cleavage intermediates. Arrows indicate the precursor-product relationship between Pr55^{Gag} and its cleavage intermediates (top panel) or the cleavage intermediates and free CA (bottom panel). The vertical dotted line indicates the window of time (0 min to 6 h) during which the most dramatic increases in Gag protein phosphorylation were detected.

thermore, we propose that the phosphorylated free CA detected in SIV virions is derived from intravirion cleavage of phosphorylated domain form CA by PR. One could argue that, instead, the phosphorylated free CA detected in virions is derived from independent phosphorylation events that occur before the 0-min time point of our assay for active phosphorylation of Gag (Fig. 5) and that intravirion cleavage of phosphorylated CA domains by PR does not contribute to the pool of phosphorylated free CA in SIV virions. However, Fig. 4 demonstrates that at least some CA domains are cleaved all the way to free CA within the virion: the total amount of free CA in virions increases over time due to PR activity. Thus, in order for intravirion cleavage of phosphorylated domain form CA to not contribute to the pool of phosphorylated free CA in virions, PR would have to cleave only nonphosphorylated CA domains all the way to free CA in the virion. If this were true, the phosphorylation of free CA over time (an indicator of changes in the CA ^{32}P/^{35}S ratio) would decrease: the numerator in the ^{32}P/^{35}S ratio (the free CA ^{32}P band intensity) would remain constant, while the denominator (the free CA ^{35}S band intensity) would increase over time. This is not the case: the phosphorylation of free CA over time does not change significantly (Fig. 5C). Therefore, we conclude that at least some of the phosphorylated free CA present in SIV virions must be derived from the intravirion cleavage of phosphorylated CA domains by PR. This result indicates that when CA domains are both phosphorylated by a virion-associated kinase and cleaved by PR in SIV virions, phosphorylation precedes the completion of cleavage.

DISCUSSION

In this study, we provide definitive evidence that proteolytic processing of Pr55^{Gag} and its cleavage intermediates can occur in budded SIV virions. Several viral proteins are known to be cleaved inside retroviral virions by PR, such as HIV-1 Vif (35), HIV-1 and HIV-2 Nef (4, 43, 50, 54), and, in several retroviruses, the cytoplasmic tail of the transmembrane portion of Env (2, 19, 23). However, up to this point, it has not been generally agreed that Gag proteins can be cleaved in budded virus, with the exception of the CA-p2 intermediate of Rous sarcoma virus Gag (1). In fact, there are conflicting reports in the literature as to whether Gag processing occurs in HIV-1 virions. Ten years ago, one group was unable to detect changes in Gag protein levels over time in HIV-1 virions using an assay quite similar to the one used in this study with the exception that changes in Gag protein levels were assayed by immunoblotting (29). As such, it was believed that, in HIV-1, processing must be completed either before the virion is released from the cell or in a very small window of time (less than 10 s) after the virion has been released (29). In contrast, in their recent studies of intravirion Vif cleavage, Khan and colleagues observed cleavage of Pr55^{Gag} in HIV-1 virions over a period of several hours postbudding (35), similar to our findings in SIV. In our view, the apparently contradictory results in HIV-1 can be explained by differences in experimental design: ^{35}S labeling of virus (performed as described in reference 35 and this study) is necessary to attain the level of sensitivity required to detect subtle changes in Gag protein levels over time. Similar assays using other retroviruses will ultimately be required to deter-

mine whether intravirion cleavage of $Pr55^{Gag}$ and its intermediates by PR is specific to HIV and SIV or conserved among retroviruses.

Two of our findings indicate that active phosphorylation of CA-containing Gag proteins occurs predominantly, if not exclusively, in budded SIV virions: (i) phosphorylated forms of $Pr55^{Gag}$, three of its CA-containing cleavage intermediates (p50, p41, and CA-p2), and free CA are detectable in budded virions but not in virus-producing cells (Fig. 1) and (ii) $Pr55^{Gag}$ and the same three cleavage intermediates are actively phosphorylated over time in budded virus (Fig. 5). These findings also indicate that at least one cellular kinase capable of Gag phosphorylation is incorporated into SIV virions. CA is clearly a substrate for this virion-associated kinase: the observed phosphorylation of CA-p2 over time in budded virions (Fig. 5) must occur on the CA domain, and (although we cannot be certain) it is possible that the phosphorylation of $Pr55^{Gag}$, p50, and p41 over time occurs on the CA domains of these proteins as well. However, as free CA is not actively phosphorylated over time in budded virions, the virion-associated kinase clearly prefers domain forms of CA to free CA as a substrate. Although the identity of the virion-associated kinase(s) responsible for phosphorylation of SIV CA remains to be determined, several kinases that are known or suspected to phosphorylate Gag proteins are incorporated into HIV virions: the catalytic subunit of cellular protein kinase A (C-PKA) (8), active extracellular signal-regulated kinase 2 (ERK-2) (7, 28), and a 53-kDa serine/threonine kinase that remains to be identified (7). Virion-associated kinase activity appears to be a common feature of enveloped viruses (reviewed in reference 26), making it tempting to speculate that virion-associated kinases play an important role in the life cycle of enveloped viruses.

If, as our data suggest, cleavage of phosphorylated domain form CA by PR is the source of phosphorylated free CA (Fig. 5), why is domain form CA more phosphorylated than free CA (Fig. 3)? In our view, there are two possible explanations: (i) there is a pool of hyperphosphorylated domain form CA that is not cleaved to free CA by SIV PR or (ii) the domain form of CA is phosphorylated by a virion-associated kinase and then dephosphorylated by a virion-associated phosphatase before the completion of CA cleavage. Identification of the kinase(s) and/or phosphatase(s) incorporated into SIV virions would greatly facilitate determination of which of these scenarios occurs in budded virus.

We have demonstrated that both phosphorylation and cleavage of CA-containing Gag proteins can occur in budded SIV virions. It should be noted that cleavage of Gag proteins was detected within only the first 6 h of our time course assays (Fig. 4) and that the most dramatic increases in Gag protein phosphorylation were detected within the same window of time (Fig. 5). These early time points may in fact be the most biologically relevant, as several factors may contribute to a decline in virion infectivity over time. Therefore, it is quite possible that the phosphorylation and proteolysis of Gag that we detect in our in vitro assays represent Gag modifications that are functionally important for virus replication in vivo.

Based on the studies presented in this paper, we propose the following model for the phosphorylation and cleavage of CA-containing Gag proteins in the late stages of the SIV life cycle. Gag proteins assemble at the plasma membrane of the cell, and PR initiates Gag cleavage inside the virion as it buds. One or more cellular kinases are incorporated into the virion during this assembly and budding process. Once the virion is released from the cell, the incorporated kinase begins to phosphorylate CA domains in the Gag polyproteins ($Pr55^{Gag}$ and several of its intermediates) that have not yet been cleaved by PR. PR then cleaves some of these phosphorylated CA domains all the way to free CA.

As discussed, the most dramatic increases in phosphorylation of CA-containing Gag proteins were detected in the same window of time during which all of the cleavage of these proteins by PR was detected (0 min to 6 h). This observation, coupled with studies of Gag proteins in other retroviruses which indicate that phosphorylation of Gag stimulates its cleavage by PR (41, 55), leads us to hypothesize that the phosphorylation and proteolysis of Gag proteins may be functionally linked in SIV. Since we also demonstrated that, in budded virions, the phosphorylation of CA domains in $Pr55^{Gag}$ and/or its cleavage intermediates precedes the cleavage of these domains by the viral protease (Fig. 5), it is possible that phosphorylation of CA domains regulates the timing of either Gag cleavage by PR or virion maturation in SIV. Efforts focused on identifying the site(s) of CA phosphorylation should clarify the function of this modification in the life cycle of SIV.

ACKNOWLEDGMENTS

We thank Robert Cole for his contributions as Director of the AB Mass Spectrometry/Proteomics Facility at the Johns Hopkins University School of Medicine (www.hopkinsmedicine.org/msf/), which is supported by a National Center for Research Resources shared instrumentation grant (1S10-RR14702), the Johns Hopkins Fund for Medical Discovery, and the Institute for Cell Engineering. We also thank Jef Boeke, Carolyn Machamer, and James Hildreth for insightful discussions. The reagents p239SpSp5′ and p239SpE3′ from Ronald Desrosiers and HIV-2 protease from Bret Shirley and Michael Cappola, Boehringer Ingelheim Pharmaceuticals, Inc., were obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

This work was supported by grants to J.E.C. from the National Institutes of Health (NS35751, NS38008, MH70306, and NS47984).

REFERENCES

1. **Bennett, R. P., S. Rhee, R. C. Craven, E. Hunter, and J. W. Wills.** 1991. Amino acids encoded downstream of *gag* are not required by Rous sarcoma virus protease during Gag-mediated assembly. J. Virol. **65:**272–280.
2. **Brody, B. A., S. S. Rhee, M. A. Sommerfelt, and E. Hunter.** 1992. A viral protease-mediated cleavage of the transmembrane glycoprotein of Mason-Pfizer monkey virus can be suppressed by mutations within the matrix protein. Proc. Natl. Acad. Sci. USA **89:**3443–3447.
3. **Bruett, L., and J. E. Clements.** 2001. Functional murine leukemia virus vectors pseudotyped with the visna virus envelope show expanded visna virus cell tropism. J. Virol. **75:**11464–11473.
4. **Bukovsky, A. A., T. Dorfman, A. Weimann, and H. G. Gottlinger.** 1997. Nef association with human immunodeficiency virus type 1 virions and cleavage by the viral protease. J. Virol. **71:**1013–1018.
5. **Bukrinskaya, A. G., A. Ghorpade, N. K. Heinzinger, T. E. Smithgall, R. E. Lewis, and M. Stevenson.** 1996. Phosphorylation-dependent human immunodeficiency virus type 1 infection and nuclear targeting of viral DNA. Proc. Natl. Acad. Sci. USA **93:**367–371.
6. **Burnette, B., G. Yu, and R. L. Felsted.** 1993. Phosphorylation of HIV-1 Gag proteins by protein kinase C. J. Biol. Chem. **268:**8698–8703.
7. **Cartier, C., M. Deckert, C. Grangeasse, R. Trauger, F. Jensen, A. Bernard, A. Cozzone, C. Desgranges, and V. Boyer.** 1997. Association of mitogen-activated protein kinase with human immunodeficiency virus particles. J. Virol. **71:**4832–4837.
8. **Cartier, C., B. Hemonnot, B. Gay, M. Bardy, C. Sanchiz, C. Devaux, and L. Briant.** 2003. Active cAMP-dependent protein kinase incorporated within highly purified HIV-1 particles is required for viral infectivity and interacts with viral capsid protein. J. Biol. Chem. **278:**35211–35219.

9. **Cartier, C., P. Sivard, C. Tranchat, D. Decimo, C. Desgranges, and V. Boyer.** 1999. Identification of three major phosphorylation sites within HIV-1 capsid. Role of phosphorylation during the early steps of infection. J. Biol. Chem. **274:**19434–19440.
10. **di Marzo Veronese, F., T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1988. Biochemical and immunological analysis of human immunodeficiency virus *gag* gene products p17 and p24. J. Virol. **62:**795–801.
11. **Freed, E. O.** 1998. HIV-1 Gag proteins: diverse functions in the virus life cycle. Virology **251:**1–15.
12. **Gallay, P., S. Swingler, C. Aiken, and D. Trono.** 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. Cell **80:**379–388.
13. **Gatlin, J., S. J. Arrigo, and M. G. Schmidt.** 1998. HIV-1 protease regulation: the role of the major homology region and adjacent C-terminal capsid sequences. J. Biomed. Sci. **5:**305–308.
14. **Gatlin, J., S. J. Arrigo, and M. G. Schmidt.** 1998. Regulation of intracellular human immunodeficiency virus type-1 protease activity. Virology **244:**87–96.
15. **Gelderblom, H. R.** 1991. Assembly and morphology of HIV: potential effect of structure on viral function. AIDS **5:**617–637.
16. **Gelderblom, H. R., E. H. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **156:**171–176.
17. **Gelderblom, H. R., M. Ozel, and G. Pauli.** 1989. Morphogenesis and morphology of HIV. Structure-function relations. Arch. Virol. **106:**1–13.
18. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**5781–5785.
19. **Green, N., T. M. Shinnick, O. Witte, A. Ponticelli, J. G. Sutcliffe, and R. A. Lerner.** 1981. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. Proc. Natl. Acad. Sci. USA **78:**6023–6027.
20. **Hemonnot, B., C. Cartier, B. Gay, S. Rebuffat, M. Bardy, C. Devaux, V. Boyer, and L. Briant.** 2004. The host cell MAP kinase ERK-2 regulates viral assembly and release by phosphorylating the p6gag protein of HIV-1. J. Biol. Chem. **279:**32426–32434.
21. **Henderson, L. E., R. Benveniste, R. Sowder, T. D. Copeland, A. M. Schultz, and S. Oroszlan.** 1988. Molecular characterization of *gag* proteins from simian immunodeficiency virus (SIV_{Mne}). J. Virol. **62:**2587–2595.
22. **Henderson, L. E., T. D. Copeland, R. C. Sowder, A. M. Schultz, and S. Oroszlan.** 1988. Analysis of proteins and peptides purified from sucrose gradient banded HTLV-III. Alan R. Liss Inc., New York, N.Y.
23. **Henderson, L. E., R. Sowder, T. D. Copeland, G. Smythers, and S. Oroszlan.** 1984. Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described *gag* and *env* cleavage products. J. Virol. **52:**492–500.
24. **Henderson, L. E., R. C. Sowder, T. D. Copeland, S. Oroszlan, and R. E. Benveniste.** 1990. Gag precursors of HIV and SIV are cleaved into six proteins found in the mature virions. J. Med. Primatol. **19:**411–419.
25. **Hoglund, S., L. G. Ofverstedt, A. Nilsson, P. Lundquist, H. Gelderblom, M. Ozel, and U. Skoglund.** 1992. Spatial visualization of the maturing HIV-1 core and its linkage to the envelope. AIDS Res. Hum. Retrovir. **8:**1–7.
26. **Hui, E. K.** 2002. Virion-associated protein kinases. Cell. Mol. Life Sci. **59:**920–931.
27. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature **331:**280–283.
28. **Jacque, J. M., A. Mann, H. Enslen, N. Sharova, B. Brichacek, R. J. Davis, and M. Stevenson.** 1998. Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase. EMBO J. **17:**2607–2618.
29. **Kaplan, A. H., M. Manchester, and R. Swanstrom.** 1994. The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency. J. Virol. **68:**6782–6786.
30. **Kaplan, A. H., and R. Swanstrom.** 1991. The HIV-1 gag precursor is processed via two pathways: implications for cytotoxicity. Biomed. Biochim. Acta **50:**647–653.
31. **Kaplan, A. H., and R. Swanstrom.** 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. Proc. Natl. Acad. Sci. USA **88:**4528–4532.
32. **Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1993. Partial inhibition of the human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. **67:**4050–4055.
33. **Katsumoto, T., M. Asanaka, S. Kageyama, T. Kurimura, K. Nakajima, A. Noto, H. Tanaka, and R. Sato.** 1990. Budding process and maturation of human immunodeficiency virus examined by means of pre- and post-embedding immunocolloidal gold electron microscopy. J. Electron Microsc. **39:**33–38.
34. **Kestler, H., T. Kodama, D. Ringler, M. Marthas, N. Pedersen, A. Lackner, D. Regier, P. Sehgal, M. Daniel, N. King, et al.** 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science **248:**1109–1112.
35. **Khan, M. A., H. Akari, S. Kao, C. Aberham, D. Davis, A. Buckler-White, and K. Strebel.** 2002. Intravirion processing of the human immunodeficiency virus type 1 Vif protein by the viral protease may be correlated with Vif function. J. Virol. **76:**9112–9123.
36. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85:**4686–4690.
37. **Kramer, R. A., M. D. Schaber, A. M. Skalka, K. Ganguly, F. Wong-Staal, and E. P. Reddy.** 1986. HTLV-III gag protein is processed in yeast cells by the virus pol-protease. Science **231:**1580–1584.
38. **Laurent, A. G., B. Krust, M. A. Rey, L. Montagnier, and A. G. Hovanessian.** 1989. Cell surface expression of several species of human immunodeficiency virus type 1 major core protein. J. Virol. **63:**4074–4078.
39. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. R. Salazar, H. W. Chan, and S. Venkatesan.** 1988. The *gag* gene products of human immunodeficiency virus type 1: alignment within the *gag* open reading frame, identification of posttranslational modifications, and evidence for alternative *gag* precursors. J. Virol. **62:**3993–4002.
40. **Muller, B., T. Patschinsky, and H. G. Krausslich.** 2002. The late-domain-containing protein p6 is the predominant phosphoprotein of human immunodeficiency virus type 1 particles. J. Virol. **76:**1015–1024.
41. **Naso, R. B., W. L. Karshin, Y. H. Wu, and R. B. Arlinghaus.** 1979. Characterization of 40,000- and 25,000-dalton intermediate precursors to Rauscher murine leukemia virus *gag* gene products. J. Virol. **32:**187–198.
42. **Palmer, E., C. Sporborg, A. Harrison, M. L. Martin, and P. Feorino.** 1985. Morphology and immunoelectron microscopy of AIDS virus. Arch. Virol. **85:**189–196.
43. **Pandori, M. W., N. J. Fitch, H. M. Craig, D. D. Richman, C. A. Spina, and J. C. Guatelli.** 1996. Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. J. Virol. **70:**4283–4290.
44. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63:**2550–2556.
45. **Pettit, S. C., M. D. Moody, R. S. Wehbie, A. H. Kaplan, P. V. Nantermet, C. A. Klein, and R. Swanstrom.** 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J. Virol. **68:**8017–8027.
46. **Pettit, S. C., N. Sheng, R. Tritch, S. Erickson-Viitanen, and R. Swanstrom.** 1998. The regulation of sequential processing of HIV-1 Gag by the viral protease. Adv. Exp. Med. Biol. **436:**15–25.
47. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retrovir. **6:**1221–1231.
48. **Rittenhouse, J., M. C. Turon, R. J. Helfrich, K. S. Albrecht, D. Weigl, R. L. Simmer, F. Mordini, J. Erickson, and W. E. Kohlbrenner.** 1990. Affinity purification of HIV-1 and HIV-2 proteases from recombinant E. coli strains using pepstatin-agarose. Biochem. Biophys. Res. Commun. **171:**60–66.
49. **Rue, S. M., J. W. Roos, L. M. Amzel, J. E. Clements, and S. A. Barber.** 2003. Hydrogen bonding at a conserved threonine in lentivirus capsid is required for virus replication. J. Virol. **77:**8009–8018.
50. **Schorr, J., R. Kellner, O. Fackler, J. Freund, J. Konvalinka, N. Kienzle, H. G. Krausslich, N. Mueller-Lantzsch, and H. R. Kalbitzer.** 1996. Specific cleavage sites of Nef proteins from human immunodeficiency virus types 1 and 2 for the viral proteases. J. Virol. **70:**9051–9054.
51. **Theoretical Biology and Biophysics Group.** 2002. HIV Sequence Compendium. Theoretical Biology and Biophysics Group, Los Alamos, N.M.
52. **Tritch, R. J., Y.-S. E. Cheng, F. H. Yin, and S. Erickson-Viitanen.** 1991. Mutagenesis of protease cleavage sites in the human immunodeficiency virus type 1 *gag* polyprotein. J. Virol. **65:**922–930.
53. **Vogt, V. M.** 1996. Proteolytic processing and particle maturation. Curr. Top. Microbiol. Immunol. **214:**95–131.
54. **Welker, R., H. Kottler, H. R. Kalbitzer, and H. G. Krausslich.** 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology **219:**228–236.
55. **Yoshinaka, Y., and R. B. Luftig.** 1982. In vitro phosphorylation of murine leukemia virus proteins: specific phosphorylation of Pr65gag, the precursor of the internal core antigens. Virology **116:**181–195.