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The p2 Domain of Human Immunodeficiency Virus Type 1 Gag Regulates Sequential Proteolytic Processing and Is Required To Produce Fully Infectious Virions

STEVE C. PETTIT,[1] MARK D. MOODY,[1]† ROBERT S. WEHBIE,[1] ANDREW H. KAPLAN,[1]‡ PASCALE V. NANTERMET,[1]§ CHRISTINE A. KLEIN,[1]‖ AND RONALD SWANSTROM[1,2]*

UNC Lineberger Comprehensive Cancer Center[1] and the Department of Biochemistry and Biophysics,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

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The proteolytic processing sites of the human immunodeficiency virus type 1 (HIV-1) Gag precursor are cleaved in a sequential manner by the viral protease. We investigated the factors that regulate sequential processing. When full-length Gag protein was digested with recombinant HIV-1 protease in vitro, four of the five major processing sites in Gag were cleaved at rates that differ by as much as 400-fold. Three of these four processing sites were cleaved independently of the others. The CA/p2 site, however, was cleaved approximately 20-fold faster when the adjacent downstream p2/NC site was blocked from cleavage or when the p2 domain of Gag was deleted. These results suggest that the presence of a C-terminal p2 tail on processing intermediates slows cleavage at the upstream CA/p2 site. We also found that lower pH selectively accelerated cleavage of the CA/p2 processing site in the full-length precursor and as a peptide primarily by a sequence-based mechanism rather than by a change in protein conformation. Deletion of the p2 domain of Gag results in released virions that are less infectious despite the presence of the processed final products of Gag. These findings suggest that the p2 domain of HIV-1 Gag regulates the rate of cleavage at the CA/p2 processing site during sequential processing in vitro and in infected cells and that p2 may function in the proper assembly of virions.

Human immunodeficiency virus type 1 (HIV-1), like other retroviruses, contains the open reading frames *gag*, *pol*, and *env*. The *gag* and *pol* genes are expressed as the polyprotein precursors Pr55 (Gag) and Pr160 (Gag/Pol). Gag and Gag/Pol are subsequently cleaved by the viral protease (PR) to yield mature protein products (reviewed in reference 37). Proteolytic processing of the 55-kDa Gag precursor generates the structural proteins p17 (matrix [MA]), p24 (capsid [CA]), p7 (nucleocapsid [NC]), and p6 (17, 61). The *pol* gene is expressed by a ribosomal frameshift mechanism that occurs near the carboxyl terminus of *gag* (20). Proteolytic processing of the Pr160 Gag/Pol precursor yields the viral enzymes PR (p11), reverse transcriptase/RNase H, and integrase as well as the Gag structural proteins (29, 60). In addition, two smaller polypeptides with unknown function, p2 and p1, are generated by cleavage of the Gag precursor (17). p2 in the HIV-1 HXB2 isolate (44) is 14 amino acids long and is located between CA and NC. p1 is 16 amino acids long and is located between NC and p6.

The proteolytic processing sites in the Gag and Gag/Pol precursors are cleaved at different rates in infected cells and in vitro. For the Gag precursor, the different rates of cleavage at the processing sites result in sequential processing during which discrete intermediates appear transiently before the final protein products (12, 34, 38, 58, 62). Three determinants are likely to control the ordered proteolytic processing of the Gag precursor: (i) the sequence of the processing site, (ii) the structural context of the processing site, and (iii) the accessibility of the site to the protease. The diversity of the processing site sequences contributes to sequential processing, because peptides representing the known HIV-1 Gag processing sites are cleaved at different rates (3, 6, 26, 55). However, there is little correlation between the favorable kinetic parameters of peptide substrates representative of the Gag sites and the order of cleavage observed in infected cells. This suggests that additional determinants, such as structure and accessibility, influence sequential Gag processing (26, 52, 54, 58), although their influence has not been defined. The importance of ordered Gag processing in the production of a mature, infectious virion is unknown. Ordered cleavage of the viral protein precursors may be an essential component of core condensation. It is clear that processing must occur at each of the Gag sites and to near completion to produce virions with normal core morphology (11, 22).

The function of the small peptide domains p2 and p1 of the HIV-1 Gag precursor is also unknown. Similar domains are found in diverse retroviruses such as mouse mammary tumor virus, Rous sarcoma virus, bovine leukemia virus, and other lentiviruses. In the primate lentiviruses, p2 and p1 are conserved in location in the Gag precursor, but they are poorly conserved in sequence and length (16). In spite of the lack of sequence conservation, the conservation of location suggests that they have an important function in the retrovirus life cycle. In several cases, these small peptide domains are further cleaved by the viral PR after being released from the Gag precursor, suggesting they function transiently during sequential processing or core formation as a component of a larger intermediate (17, 39).

To examine the factors that regulate sequential processing of HIV-1 Gag, the full-length Gag precursor was processed by the viral PR in vitro. Under these conditions, Gag is processed

* Corresponding author. Phone: (919) 966-5710. Fax: (919) 966-3015.
† Present address: Gene-Trak Systems, Framingham, MA 01701.
‡ Present address: Departments of Medicine and Microbiology and Immunology, University of California at Los Angeles School of Medicine, Los Angeles, CA 90024.
§ Present address: Department of Immunology, Scripps Institute, La Jolla, CA 92037.
‖ Present address: Cadus Pharmaceutical Corp., New York, NY 10014.

in a sequential manner to yield intermediates and final products similar to those observed in infected cells (10, 26, 38, 58). First, we examined whether the rate of cleavage at four different Gag processing sites is influenced by the extent of cleavage at the other sites. Second, the relative contributions of processing site sequence and context in influencing the rate of cleavage were evaluated by placement of two sites that vary widely in their rates of cleavage in the same context in the Gag precursor and by the hydrolysis of peptide substrates. Third, we examined the influence of the p2 domain of Gag on sequential processing in vitro and in infected cells. Fourth, the need for p2 in the production of infectious virions was examined.

MATERIALS AND METHODS

Plasmid constructions. The phagemid used for mutagenesis, sequencing, and in vitro transcription of the *gag* gene was pGagS. pGagS contains the *gag* gene from HIV-1 isolate HXB2 (44) inserted into the vector pIBI20 (International Biotechnologies). Features of pGagS include the *gag* gene under transcriptional control of a bacteriophage T7 promoter for the synthesis of mRNA in vitro (33) and a bacteriophage f1 origin of replication for the production of single-stranded DNA for use in site-directed mutagenesis and DNA sequencing (63).

Design of mutagenic DNA oligonucleotides and the production of mutations in the HIV-1 *gag* gene. Single-stranded uracil-substituted pGagS used in mutagenesis was prepared by infecting *dut ung* mutant *Escherichia coli* CJ236/pCJ105(Cm^r) (27) containing pGagS with M13K07(b) helper phage (63). To produce site-directed mutations in the codon for the P1 amino acid of each of the four major processing sites in *gag*, 30-base oligonucleotides complementary to single-stranded pGagS were synthesized. During oligonucleotide synthesis, all four deoxynucleotides were inserted at each nucleotide position of the codon of the P1 amino acid to generate random mutations. Synthetic 30-base oligonucleotides used for the deletion of the p2 protein domain of Gag had the following sequences: Δp2-composite, AAG GCA AGA GTT TTG ATG CAG AGA GGC AAT 3′; Δp2-fast, 5′ GGA CCC GGC CAT AAG GCT ACC ATA ATG ATG 3′; Δp2-slow, GTT TTG GCT GAA GCA GGC AAT TTT AGG AAC 3′. In vitro mutagenesis reactions and preparation of pGagS libraries containing mutations were performed as previously described (1, 27, 30, 42). Mutations were identified by DNA sequence analysis by the dideoxy chain termination method with modified T7 DNA polymerase (U.S. Biochemicals) (47).

Synthesis of 5′ capped mRNA and translation in vitro. Capped mRNA was synthesized in vitro from pGagS DNA or pGagS DNA containing selected mutations. A typical mRNA synthesis reaction mixture contained 1.0 μg of pGagS DNA, linearized with *Sal*I, in a 20-μl reaction mixture containing 500 μM (each) UTP, CTP, and ATP; 200 μM GTP; 300 μM $m^7G(5')pppG(5')G$ (Pharmacia); 40 mM Tris-HCl (pH 7.9); 6 mM $MgCl_2$; 10 mM dithiothreitol; 2 mM spermidine; 100 μg of bovine serum albumin per ml; 15 U of T7 RNA polymerase; and 40 U of RNasin ribonuclease inhibitor (Promega Corporation) (33). Transcription reaction mixtures were incubated at 37°C for 1 h, followed by a 15-min incubation with RNase-free DNase. Synthetic mRNA (~1.0 μg) was translated in a 50-μl reaction mixture of rabbit reticulocyte lysate (Promega Corporation) in the presence of 50 μCi of [^{35}S]cysteine (1,000 Ci/mM) (DuPont NEN) for 2 h at 30°C according to the manufacturer's instructions (Promega Corporation).

Expression and purification of the HIV-1 PR from *E. coli*. Recombinant HIV-1 PR used for processing of the HIV-1 Gag precursor and peptide substrates in vitro was expressed in *E. coli* BL21(DE3) cells by inserting the gene for HIV-1 protease with an initiating methionine into a bacteriophage T7 expression system (51). Following induction of expression, PR was purified by a modification of the procedure of Cheng et al. (4). Isolated inclusion bodies were solubilized in 10 ml of a buffer containing 8 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.5), and 5 mM dithiothreitol and clarified by centrifugation. The solubilized protein was filtered with a Centriprep-100 ultrafiltration device (Amicon) at 5,000 × *g*. The protein in the filtrate was precipitated by the addition of 10 volumes of methanol, placed at −20°C for 1 h, and collected by centrifugation at 25,000 × *g* for 15 min. The pellet was resuspended in 3 ml of a buffer containing 8 M urea, 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, and 2 mM EDTA. The concentration of protein in the 8 M urea solution was determined by using a colorimetric assay (Bio-Rad Laboratories), and the volume of the solution was adjusted to a concentration of less than 1 mg/ml. PR was refolded by rapid dilution into 20 volumes of ice-cold folding buffer (20 mM sodium acetate [pH 5.5], containing 1 mM EDTA, 2.5 mM dithiothreitol, and 10% glycerol) (53). After refolding, any insoluble aggregated protein was removed by centrifugation at 25,000 × *g* for 15 min. The refolded PR was concentrated in a Centriprep-10 filtration device (Amicon) at 4°C, during which time the buffer was exchanged with 3 volumes of fresh folding buffer to remove any residual urea. Aliquots of the refolded PR were stored at −70°C. PR obtained by this procedure was greater than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and (28) was stable for several months when stored at −70°C.

In vitro assays for the proteolytic processing of Gag. Processing of the Gag precursor in vitro was performed with 50-μl reaction mixtures containing 5 μl of the rabbit reticulocyte lysate translation mixture and purified recombinant PR (1.0 to 5.0 μg) in modified AMT buffer (9) (50 mM acetic acid, 50 mM MES [morpholineethanesulfonic acid], 100 mM Tris, 150 mM NaCl) at either pH 5.0 or pH 7.0. All reactions were performed at 30°C. Aliquots of 5 μl were taken at various times, mixed with 10 μl of 2× SDS-PAGE loading buffer (28), and heated to 100°C for 3 min. Products of the processing reaction were separated by SDS-PAGE (28), fixed in 20% methanol–10% acetic acid–5% glycerol, dried, and exposed to X-Omat AR film (Eastman Kodak Co.). Relative quantitation of labeled protein products was performed by densitometry analysis of autoradiographs on a Molecular Dynamics model 300 densitometer or by analysis of the radioactivity in the gel by using a Molecular Dynamics model 400 Phosphoimager.

Calculation of the relative rates of cleavage for the processing sites in Gag. The relative amount of protein represented by bands in autoradiographs of Gag processing assays was determined by correcting for the relative amount of cysteine label in each protein species. The amount of uncleaved substrate for an individual site was determined as the remaining amount of the various precursors containing the uncleaved site. This value was represented as a percentage of the total amount of substrate in the lane by dividing by the total amount of p24-containing species. In some instances the amount of some intermediates could not be determined because of background radioactivity in the size range of 7 to 10 kDa that appeared on the autoradiographs. In these cases, the indiscernible intermediates were not included in the calculations of total uncleaved substrate.

Relative rates of cleavage for the individual sites in the Gag precursor were calculated from semi-log (first-order) plots in which the log of the percentage of the initial substrate remain-

ing was plotted versus time. The rate of cleavage at a particular site was estimated by the average slope of the line through time points representing 20 to 80% cleavage. The maximum amount of assay time utilized for the rate calculations was 1 h, during which time the PR retained at least 80% of its initial activity as judged by cleavage of a radiolabeled peptide substrate under identical assay conditions. The numerical values of the rate were then used to compare the relative rates of cleavage for the Gag processing sites.

Construction of infectious HIV-1 clones, cell transfection, and Western blots (immunoblots). Regions of the plasmid pGagS containing deletions in the p2 domain of Gag were subcloned into the plasmid pMM4, which contains an infectious genomic copy of the HIV-1 HXB2 isolate (44). An aliquot of 30 μg of twice-CsCl-banded plasmid DNA was transfected into HeLa cells in 100-mm plates by $CaPO_4$ precipitation (13, 21). At 48 h posttransfection, the medium was collected and stored frozen for later use. The cells were washed in phosphate-buffered saline and lysed in 300 μl of modified radioimmunoprecipitation assay buffer (21), with 100 μg of AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] per ml (ICN Biomedicals) replacing phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation at 15,000 × *g* for 10 min. For Western blot analysis, 3 to 15 μl of extract was subjected to SDS-PAGE and transblotted to nitrocellulose as described previously (41). Western blotting was performed with biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate as described previously (41) with the following modification: blots were blocked in 0.5% gelatin in Tris-buffered saline–0.05% Tween 20 (TBS-Tw) for 1 h. Blocked blots were exposed to either anti-HIV-1 matrix monoclonal antibody (Cellular Products, Inc.) or anti-HIV-1 capsid monoclonal antibody (DuPont) at a 1:1,000 dilution in TBS-Tw, or were exposed to anti-HIV-1 p6 monoclonal antibody (61) at a 1:30 dilution in TBS-Tw.

Determination of the production and relative infectivity of wild-type and mutated virions. The presence of virus particles released into the medium was determined by the sedimentation of particles through a cushion of 15% sucrose (100,000 × *g*, 90 min) (5, 64). The presence of virus particles in the medium and pellet was confirmed with a reverse transcriptase assay (36) and a p24 CA antigen enzyme-linked immunosorbent assay (ELISA) (DuPont). In addition, the presence of the processed Gag proteins MA, CA, and p6 in the pelleted virions was confirmed by Western blotting.

The ability of released virus to infect cells was judged by two methods. First, virus-containing medium was used to infect CD4-LTR/β-gal indicator cells (Magi cells) as described previously (24). Two days later, infected cells were detected by colorimetric assay for evidence of infection. The relative infectivity of the released virus particles was estimated by comparing the number of infectious units as measured on CD4-LTR/β-gal cells with the amount of virion-associated p24 CA antigen in the medium as determined by ELISA. In the second assay, we determined the ability of released virus particles to productively infect CEM cells. A sample of 2×10^6 cells was exposed to 500 μl of virus-containing medium collected 48 h posttransfection as described previously (21). The cells were observed daily for the presence of cytopathic effect. After 3 weeks, all cultures were assayed for the presence of p24 CA antigen in the medium by using an ELISA.

Determination of the PR activity on peptide substrates. PR activity was assayed in AMT buffer at 37°C either with a radiometric assay or by analysis of reaction products by fast protein liquid chromatography (FPLC). The kinetic parameters of K_m and k_{cat} (the Michaelis-Menten parameter for catalytic-site activity) values were obtained by fitting data to the Michaelis-Menten equation with a basic program employing the nonlinear regression routine of Duggleby (8), which was kindly provided by Dexter Northrop, University of Wisconsin.

The radiometric assay employed the substrate [^{3}H]Ac-SQNYPIVR in a modification of an assay described by Ido et al. (19). Reactions were performed in 25-μl volumes and were linear over the 30-min period of incubation, with less than 10% substrate cleavage. The assays were terminated by the addition of Dowex X-50W-X4 (5% [wt/vol]) in 0.1 M acetic acid. The acetylated cleavage product in the supernatant was quantitated by liquid scintillation counting.

Reactions with the peptide KARVLAEAMSNph were performed in 100 μl (pH 7.0) or 400 μl (pH 5.0) of AMT buffer and were terminated after 30 min with trifluoroacetic acid to a final concentration of 1%. The cleavage product AEAMSNph was separated from the uncleaved substrate by FPLC and quantitated spectrophotometrically. The kinetic measurements for both peptide substrates were determined at concentrations that spanned the derived K_m values except for the substrate representative of the MA/CA processing site, for which maximum solubility was obtained at 19 mM at pH 7.

RESULTS

Cleavage of the Gag precursor in vitro: observed intermediates and final products. To study the determinants that regulate sequential processing of the HIV-1 Gag precursor, we employed an assay in which labeled Gag precursor is processed sequentially in vitro after the addition of HIV-1 PR (10, 26, 38, 58). The intermediates and final products that arise during sequential processing of Gag with this assay correlate well with processing events observed in infected cells (12, 34, 38, 58, 62). The assay has advantages in that differences in the rates of cleavage at the Gag processing sites can be more accurately estimated than those in infected cells. Previously published data describing sequential processing of the HIV-1 precursor in vitro (10, 26, 38, 58), in combination with the mutagenesis of processing sites (see below), allowed the composition of processing intermediates and final products to be determined.

Translation of synthetic *gag* mRNA in a rabbit reticulocyte lysate system yielded labeled full-length Gag precursor Pr55 as the predominant protein product (Fig. 1, lanes 0 min, and Fig. 2A), which was rapidly processed at neutral pH upon addition of recombinant HIV-1 PR. Initially, Gag was cleaved at the p2/NC cleavage site to yield the MA-CA-p2 intermediate (designated p39a) and the p15 intermediate (NC-p1-p6) previously described by Veronese et al. (61) (Fig. 1, lanes 1, and Fig. 2B). The p15 intermediate was cleaved at the p1/p6 site approximately ninefold more slowly than the initial cleavage to yield a 7-kDa product (p7) consisting of NC-p1. The p39a intermediate was later cleaved at the MA/CA site at an approximately 14-fold slower rate to yield the MA final product, p17, and a p25 intermediate consisting of CA-p2. In the fourth cleavage observed, the CA/p2 site of the p25 intermediate was cleaved at an estimated 400-fold slower rate than the initial cleavage at p2/NC. In some experiments, we also observed slow processing of the NC-p1 intermediate to yield the unidentified product p7*. However, the apparent size of p7* during SDS-PAGE varied with the pH of the cleavage assay, suggesting altered processing with changes in pH (40). Thus, the order of cleavages for the four processing sites at neutral pH is p2/NC > p1/p6 ≥ MA/CA > CA/p2, with the two sites flanking the p2 domain differing roughly 400-fold in their rate of cleavage. This order is in agreement with data from

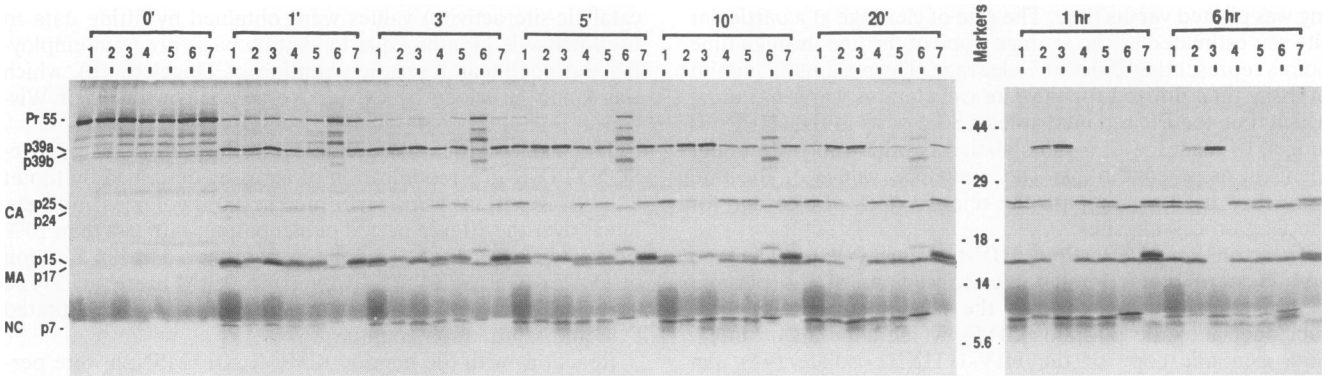

FIG. 1. Sequential processing in vitro of the HIV-1 Gag precursor and of Gag with P1 amino acid substitutions. Assays were performed with labeled Gag as described in Materials and Methods at pH 7. Aliquots were collected from the processing reaction mixtures at the times indicated and subjected to SDS-PAGE with a 17% acrylamide gel (28). Molecular mass markers and the observed intermediates and final products for wild-type Gag (lanes 1) are shown. Lanes containing aliquots from processing reactions of Gag with P1 substitutions are as follows: lanes 2, P1 Leu at the MA/CA site; lanes 3, P1 Ile at the MA/CA site; lanes 4, P1 Ile at the CA/p2 site; lanes 5, P1 Leu at the p2/NC site; lanes 6, P1 Ile at the p2/NC site; lanes 7, P1 Ile at the p1/p6 site. The dark area that extends from 7 to 10 kDa on autoradiographs of SDS-PAGE gels was found to be an artifact of translation in vitro when [^{35}S]Cys is used as a label because it was also present in translations without added mRNA (not shown). During SDS-PAGE, the p15 intermediate was observed to migrate more slowly than p17 MA. This altered migration was previously observed for these products during processing in vitro (38) and has also been observed for viral proteins (40). The HIV-1 p6 protein (61) was not visible in the autoradiographs of SDS-PAGE gels because it contained no labeled residues. The amino acid sequence of the HIV-1 PR was as reported previously (44).

previous reports (26, 38, 58), although the differences in rate observed here are greater than earlier estimates (10, 58).

Cleavage of the processing sites in the Gag precursor are not necessarily independent events. To determine the independence of the cleavages during sequential processing, we constructed four Gag precursors containing mutations designed to prevent processing at the MA/CA, CA/p2, p2/NC, or p1/p6 site. The effect of the blocking mutation on the cleavage of the other three sites was monitored. Previous reports indicated that conservative substitution of the β-branched amino acid Ile at the P1 position of a site prevents cleavage of a Pol processing site in *E. coli* (42) (the P1 residue is the first amino acid located upstream of the scissile bond in the substrate [48]) and of peptides representative of cleavage sites (2, 25, 45, 57). As a control, the similar amino acid Leu was also substituted for the P1 amino acid at either the MA/CA or p2/NC site. Leu is common to the P1 position of processing sites (42, 43) and is the P1 amino acid of the CA/p2 processing site (17). As shown in Fig. 1 (lanes 3, 4, 6, and 7), substitution of the P1 residue with Ile prevented cleavage at each of the four sites as judged by the absence of one or more of the final processed products. Preventing cleavage at a site also altered the composition or time of appearance of intermediate products during sequential processing (Fig. 1 and 2C). P1 Leu substitutions at the MA/CA and p2/NC sites caused these sites to be cleaved at rates estimated to be 15- and 2-fold slower, respectively. The P1 Leu substitutions also resulted in the transient appearance of the same novel intermediates observed with a P1 Ile substitution (Fig. 1, lanes 2 and 5), although with a P1 Leu substitution, these intermediates were eventually cleaved. Thus, the substitution of a P1 Ile blocked cleavage at all four Gag processing sites, while a P1 Leu substitution reduced the rate of cleavage by an amount that was dependent on the site.

With one exception, cleavage of the four Gag sites occurred independently (Fig. 3). The exception occurred for the site upstream of the p2 domain. When initial cleavage at the p2/NC site was blocked, the upstream CA/p2 site was cleaved approximately 20-fold faster, without affecting the rate of cleavage at the other sites. The rate of cleavage at the CA/p2 site was not influenced by blocking the other sites in Gag. Accelerated cleavage of the CA/p2 site was not strictly dependent on the P1 Ile substitution at the p2/NC site, because other mutations that blocked cleavage at the p2/NC site also accelerated cleavage at the CA/p2 site (40). These data suggest that sequential processing is regulated, at least in part, by an interaction between the two sites flanking the p2 domain. Initial cleavage at the downstream site down-regulates the rate of cleavage at the upstream site. This negative regulation of cleavage at the CA/p2 site contributes significantly to the 400-fold difference in the cleavage rates at these two adjacent sites.

The p2 domain of Gag negatively regulates the rate at which the CA/p2 site is cleaved in vitro. To confirm that the p2 domain of Gag was responsible for the negative regulation of cleavage of the CA/p2 site, the p2 domain was deleted from the Gag precursor. The deletion (Δp2-slow) removed the p2 domain and the seven-amino-acid recognition sequence (3, 6, 35, 56) of the p2/NC cleavage site, leaving intact the sequence of the CA/p2 cleavage site (Fig. 4A). Without the presence of p2, the CA/p2 site in Δp2-slow Gag was accelerated by an amount similar to that observed earlier when the downstream p2/NC site was blocked from cleavage (M3771) (Fig. 4B). This suggests that p2 and not the CA/p2 cleavage site sequence is largely responsible for the slow rate of cleavage of the CA/p2 site during sequential processing. In addition, p2 must have a carboxyl terminus freed by prior cleavage of the downstream site to exert the negative effect on cleavage at the CA/p2 site.

To examine whether the p2 domain influences the rate of cleavage of the downstream p2/NC site, an additional p2 deletion (Δp2-fast) was constructed that retained the faster p2/NC cleavage site sequence (Fig. 4A). The deletion mutants Δp2-slow and Δp2-fast effectively placed the sequence of the sites flanking p2 in the same context in the Gag precursor. Compared with wild-type Gag, the p2/NC site in Δp2-fast was cleaved roughly threefold slower. Thus, without the influence of the p2 domain, the difference in the rates of cleavage at the two processing site sequences was reduced from approximately 400-fold to 15-fold (data not shown). These data indicate that

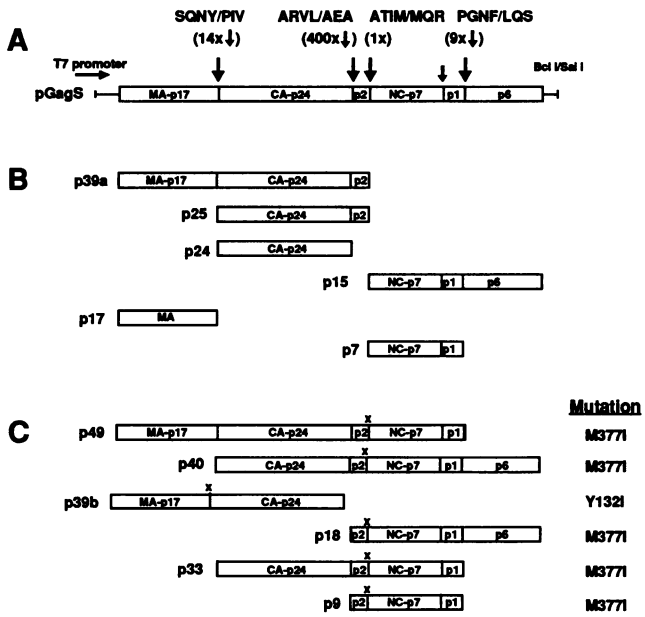

FIG. 2. HIV-1 *gag* vector and Gag processing intermediates and products. (A) The phagemid pGagS contains the HIV-1 *gag* gene under transcriptional control of the bacteriophage T7 promoter, which is utilized to produce labeled Gag precursor by transcription and translation in vitro. The locations of the five major processing sites in the Gag precursor, as identified by sequencing of virion proteins, are denoted by arrows (17). The amino acid sequences (P4 to P3′) of the four major processing sites evaluated in this study are shown in the single-letter code with the position of the scissile bond denoted by a slash. The numbers above the sites indicate differences in the estimated rate of cleavage by HIV-1 PR in vitro at pH 7, with the rate of initial cleavage at the p2/NC site set to a value of 1. (B) Schematic of processing intermediates and final products observed during sequential processing of the wild-type Gag precursor in vitro at pH 7. The intermediates and final products are shown in decreasing apparent molecular mass as determined by SDS-PAGE (28). (C) Schematic of novel processing intermediates and final products observed during sequential processing in vitro when processing sites were inhibited by a P1 Ile substitution during in vitro processing reactions at pH 7. Some of the same intermediates were observed when the assays were performed at pH 5 (see Results). The processing sites containing an inhibitory P1 Ile substitution are indicated by X. The substituted amino acid and its position within Gag translated from the sequence of the HXB2 HIV-1 isolate are indicated to the right (44).

the p2 domain in the Gag precursor functions to maximize the difference in the cleavage rate between the CA/p2 and p2/NC sites. A third p2 deletion, Δp2-composite, created a composite CA/NC site with a P1 Leu/P1′ Met combination not normally found in HIV-1 cleavage sites (31). This composite site was cleaved at a rate midway between the sites flanking p2 (data not shown).

Lower pH accelerates cleavage of the CA/p2 site by a sequence-based mechanism. Assay conditions with lower pH have been successfully used to study the accessibility of proteinase cleavage sites in native proteins (65). A previous report showed that lower pH alters the accessibility of a potential cleavage site in lactate dehydrogenase to permit cleavage by the HIV-1 PR (54). In addition most kinetic analyses of HIV-1 PR with peptide substrates are performed at the kinetic optimum, which is approximately pH 5 (3, 18, 46). We used this approach to test the possibility that protein conformation was involved in regulating the ordered cleavage of Gag.

Lowering the pH of the in vitro assay to pH 5 affected the cleavage of the four Gag sites differently. The CA/p2 site was affected the most, being cleaved roughly 20-fold faster than at pH 7. The faster cleavage at the CA/p2 site resulted in an additional 39-kDa intermediate (designated p39b), consisting of MA-CA, that migrated slightly faster than the p39a intermediate during SDS-PAGE (Fig. 5A, lane 1). Faster cleavage at the CA/p2 site also accelerated removal of p2 from the p25 CA-p2 intermediate (Fig. 5A). Cleavage at the MA/CA and p2/NC processing sites was not significantly affected, while the p1/p6 site was cleaved approximately threefold faster at lower pH. Thus, lower pH selectively accelerated cleavage of the CA/p2 site and reduced the differences in the rates of cleavage of the Gag sites to roughly 20-fold from 400-fold.

Selective acceleration of the cleavage of the CA/p2 site at lower pH apparently occurred independently of the presence of the p2 domain. We observed further acceleration in the rate of cleavage of the CA/p2 site in Gag, in which the negative effects of the p2 domain were removed by mutagenesis (M3771, Δp2-slow; Fig. 5B). The additive effects of lower pH and removing the down-regulating effects of a p2 tail resulted in over 100-fold enhancement of cleavage at the CA/p2 site in Gag with the M377I mutation and a 43-fold enhancement in Gag with the Δp2-slow mutation. These results suggest that the combination of physiological pH and negative regulation by the p2 domain is largely responsible for the slow rate of cleavage of the CA/p2 site in the virion.

Acceleration of the CA/p2 site at lower pH apparently is dependent on a component of the processing site sequence (KARVL/AEA). The sequences of the p2/NC site (ATIM/MQR) (Δp2-fast) and the CA/NC composite site sequence (KARVL/MQR) (Δp2-composite) when placed in the same context as the Δp2-slow mutation were not accelerated at pH 5 (data not shown). These results suggest the P1′ to P3′ residues of the CA/p2 site are responsible for accelerated cleavage at pH 5.

To confirm that a sequence-based mechanism is responsible for accelerated cleavage of the CA/p2 site at lower pH, peptides representative of the MA/CA and CA/p2 processing sites were digested with HIV-1 PR at pH 5 and 7 under the buffer conditions identical to those utilized for the hydrolysis of the full-length Gag precursor. The kinetic parameters of K_m and k_{cat} for hydrolysis of the two peptides are shown in Table 1. These data indicate that the K_m for both peptides was lower at pH 5 than at pH 7, in agreement with previously published reports that the K_m of hydrolysis of a peptide substrate by HIV-1 PR decreases with pH (7, 18, 19, 46). The change in the k_{cat}/K_m value with the drop in pH closely approximated the difference in the rate of cleavage between these two sites observed with the Gag precursor. The peptide representative of the CA/p2 site showed a 25-fold improvement, while the peptide representative of the MA/CA site had a 3-fold improvement. These results are consistent with the interpretation that a sequence-based mechanism is responsible for acceleration of the CA/p2 site at lower pH, rather than a change in conformation or accessibility.

The p2 domain of Gag negatively regulates cleavage of the CA/p2 site during virion formation. We examined whether the presence of the p2 domain negatively regulates the rate at which the CA/p2 site is cleaved in infected cells. To accomplish this, we transferred the Δp2-slow deletion (Fig. 4A) into an infectious molecular clone of the viral genome (44), transfected the clone containing the mutation into cells, and monitored cleavage of the CA/p2 site during sequential processing. As a control, the Δp2-composite mutation (Fig. 4A), which removes the p2 domain in its entirety without disrupting

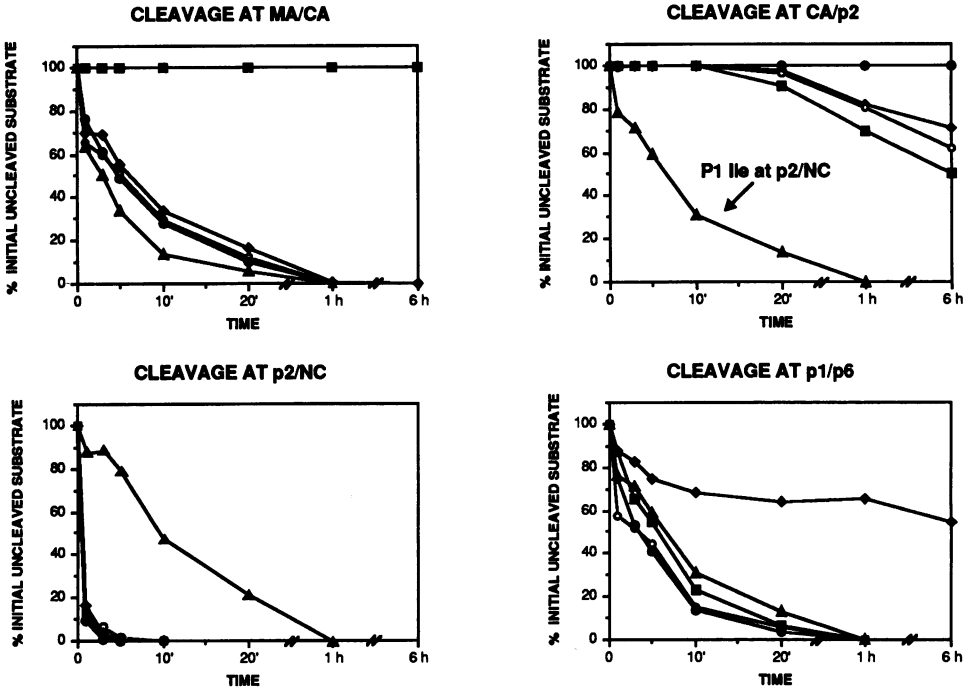

FIG. 3. The independence of cleavage at the four processing sites during sequential processing. Each plot shows the percentage of initial uncleaved substrate remaining as a function of time for cleavage at one of the four processing sites in the wild-type Gag precursor (○) and Gag in precursors containing an Ile substitution at the P1 amino acid at one of the four processing sites: MA/CA, ■; CA/p2, ●; p2/NC, ▲; and p1/p6, ◆. A P1 Ile substitution at each of the four sites prevented cleavage at that site as judged by the absence of the normal product. The apparent cleavage of substrate at the p2/NC site and p1/p6 sites containing P1 Ile substitutions is attributed to difficulties with the densitometric estimation of some intermediates comigrating with the unincorporated labeled amino acid on autoradiographs of processing reactions or the presence of secondary cleavage sites in some Gag intermediates (17).

the primary amino acid sequence of the CA or NC protein, was also transferred into an infectious viral clone. Two days posttransfection, the processing intermediates were examined by Western blotting. As shown in Fig. 6, the slow rate of cleavage at the CA/p2 site in wild-type Gag resulted in the p39b intermediate (MA/CA) being a minor species during sequential processing. In Δp2-slow Gag, however, accelerated cleavage of the CA/p2 site was demonstrated by increased amounts of the p39b intermediate. The amount of the p39b intermediate indicates that the rate of cleavage at the CA/p2 in Δp2-slow Gag is comparable to the rates of cleavage at the MA/CA site (to give p40-Δp2) and at the p1/p6 site (to give p49-Δp2 and p33). Deletion of the p2 domain, in both the Δp2-slow and Δp2-composite genomes, had no effect on the formation of the final processed products p17-MA, p24-CA, and p6 compared with the wild type, nor did the deletions have any effect on accumulation of the p15 (NC-p1-p6) intermediate. As expected, the singularly cleaved intermediates corresponding to p49 (MA-CA-p2-NC-p1) and p40 (CA-p2-NC-p1-p6) migrated faster in SDS-PAGE when the p2 domain was deleted from Gag. These results indicate that the presence of the p2 tail on processing intermediates negatively regulates the rate at which the CA/p2 site is cleaved during sequential processing in cells.

The p2 domain of Gag is important for the production of infectious virions. To determine whether the p2 domain is necessary for the budding of virions, medium from cells transfected with the wild-type genome or genomes containing either the Δp2-slow or Δp2-composite deletion mutation was examined for the presence of virus particles by sedimentation through a sucrose cushion (5, 64). As a control for the pelleting, stocks of avian leukosis virus and murine leukemia virus were also pelleted under the same conditions as HIV-1. The presence of pelleted HIV-1 virions was determined by reverse transcriptase assay, p24 CA ELISA, and Western blot analysis. As shown in Table 2, deletion of the p2 coding domain had little effect on the release of virions into the medium compared with the wild type by reverse transcriptase assay or p24 CA antigen capture (40). Western blot analysis of the pelleted virions showed predominately processed Gag proteins (MA, p24 CA, and p6) in the wild-type and p2-deleted virions (40).

Next, we examined whether virions deleted in p2 were infectious by two different methods. First, p2-deleted virions were tested for their ability to productively infect CEM cells. As shown in Table 2, virions containing either the Δp2-slow or Δp2-composite deletion of the p2 domain were not able to maintain a stable infection in CEM cells through 3 weeks of culture as measured by the presence of cytopathic effect or supernatant p24 capsid antigen. Second, we examined the ability of virus to infect cells by the multinuclear activation of a galactosidase indicator (MAGI) assay (24). The MAGI assay measures infection of a cell by the induction of an endogenous β-galactosidase gene under transcriptional control of the HIV-1 long terminal repeat and thus does not require viral spread through the culture. The titration of infectious particles by the MAGI assay, in combination with quantitation of the virion by reverse transcriptase activity, allowed the relative infectivity of virus particles to be estimated. As shown in Table 2, virus deleted in p2 was significantly less infectious. When

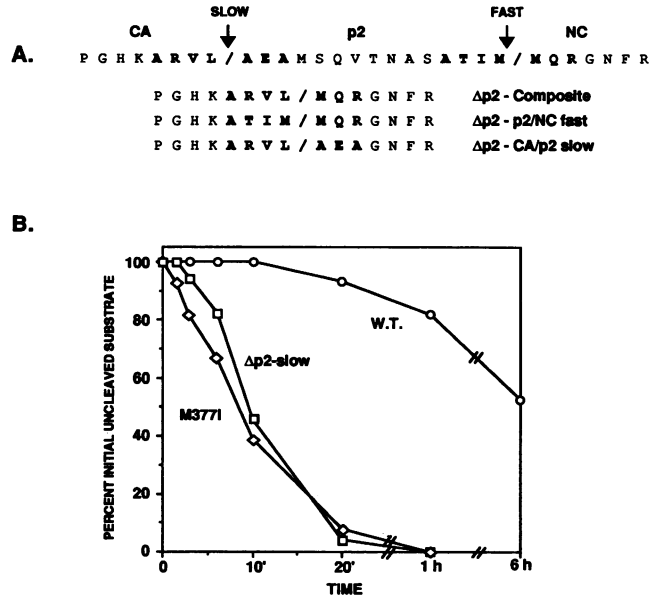

FIG. 4. The effect of the p2 domain on Gag cleavage in vitro. (A) The amino acid sequence of the p2 domain of wild-type Gag is shown in the single-letter code. The seven-amino-acid recognition sequences of the CA/p2 and p2/NC processing sites are shown in boldface type, with the slash representing the scissile bond. The p2 domain was removed in three different deletions that retained either the seven-amino-acid sequence (3, 6, 56) of the p2/NC site (Δp2-fast) or the CA/p2 site (Δp2-slow) or created a composite CA/NC processing site (Δp2-composite). (B) Accelerated cleavage of the CA/p2 site by preventing the formation of the p2 tail. The percentage of initial substrate containing an uncleaved CA/p2 site with time is shown for two mutations that prevent formation of the p2 tail during sequential processing reactions in vitro. The M3771 mutation blocked cleavage of the downstream p2/NC site by the substitution of a P1 Ile, while the Δp2-slow mutation deleted both the p2 domain and the p2/NC site from the Gag precursor. For comparison, cleavage of the wild-type precursor (W.T.) over time is also shown.

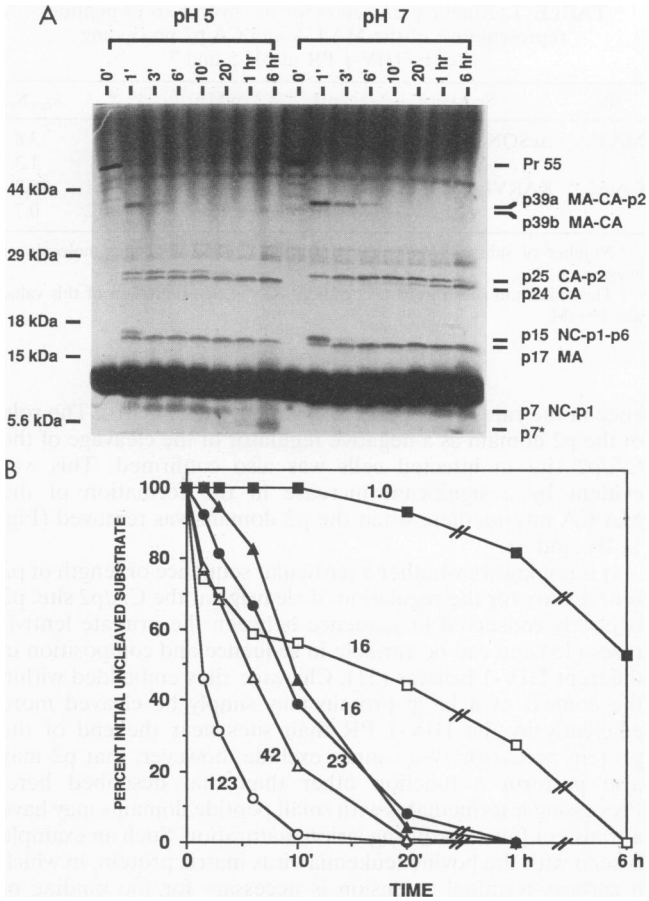

FIG. 5. Sequential processing of the HIV-1 Gag precursor in vitro at either pH 5 or pH 7. (A) Processing of labeled Gag precursor was performed as described in Materials and Methods. Aliquots were collected from the processing reaction mixtures at the times indicated. Molecular mass markers and the composition of observed intermediates and final products are shown. The estimated difference in the rates of cleavage at the two sites flanking the p2 domain was estimated to be approximately 400-fold at pH 7 and 15-fold at pH 5. (B) Accelerating effects of lower pH on the rate of cleavage at the CA/p2 processing site in wild-type Gag and Gag containing mutations preventing p2 tail formation on processing intermediates. The percentage of initial uncleaved substrate remaining with an uncleaved CA/p2 site is plotted as a function of time during sequential processing reactions. The estimated relative rates of cleavage for the CA/p2 site are also shown for a single experiment, with the rate of cleavage in the wild-type precursor set to a value of 1. ■, cleavage of the wild-type Gag precursor at pH 7; ●, Gag containing a P1 Ile substitution at p2/NC (M3771) at pH 7; ▲, Gag with both the p2 domain and the p2/NC processing site deleted (Δp2-slow) at pH 7. The corresponding open symbols indicate reactions performed at pH 5.

compared with the wild type, the Δp2-composite and the Δp2-slow deletions resulted in particles at least 20-fold less infectious. In some experiments, the Δp2-slow-deleted virus was less infectious than the Δp2-composite-deleted virus, possibly because this deletion resulted in the substitution of the first three amino acids of NC (Fig. 4A).

DISCUSSION

In this study, we have explored the mechanisms that regulate sequential processing of the HIV-1 Gag precursor. Our initial strategy was to determine if cleavage at one site was dependent on cleavage at another site. We used an approach in which each of four major processing sites was selectively blocked from cleavage by substitution of the P1 residue with Ile. A P1 Ile has been shown to block cleavage of peptide and Pol substrates by the HIV-1 PR (2, 42, 45, 57) and by the avian myeloblastosis virus PRs (25), possibly because of an unfavorable interaction of the β-branched side chain with the catalytic aspartate (57). We found that cleavage at three of the four sites examined occurred independently of cleavage at the other sites (Fig. 3). However, the CA/p2 site was accelerated 20-fold when the p2/NC site was blocked. Tritch et al. (58) have previously noted that replacement of the p2/NC cleavage site with the MA/CA processing site sequence increases the rate of cleavage at CA/p2, although the magnitude of the effect was not determined.

p2 as a regulator of sequential processing. Either mutation that blocks initial cleavage at the p2/NC site or a deletion that removes the p2 domain resulted in accelerated cleavage at the CA/p2 site (Fig. 4B). Thus, it is the presence of the p2 tail on processing intermediates (p39a and p25) that negatively regulates the rate of cleavage. These data support a model in which the p2 domain functions to regulate sequential processing by slowing cleavage at the CA/p2 site. This regulation, in turn, contributes significantly to the approximately 400-fold differ-

TABLE 1. Kinetic parameters for the hydrolysis of peptides representative of the MA/CA and CA/p2 processing sites by HIV-1 PR at pH 5 and 7

Site	Sequence	pH	K_m (mM)	k_{cat}[a]	k_{cat}/K_m
MA/CA	AcSQNY/PIVR	5.0	5.3 ± 1.4	20 ± 3	3.8
		7.0	87 ± 16[b]	114 ± 18	1.3
CA/p2	KARVL/AEAMSNph	5.0	0.11 ± 0.02	2.0 ± 0.1	18.2
		7.0	3.14 ± 0.46	2.2 ± 0.2	0.7

[a] Number of substrate molecules transformed per second per molecule of enzyme.
[b] The maximum substrate concentration used in determination of this value was 19 mM.

TABLE 2. Effect of deletion of the p2 domain on production of infectious HIV-1 particles

Virus construct	% of virion-associated RT activity in[a]:		Relative % of infectivity (wild type = 100%) in[b]:		CEM cell cytopathic effect[c]	CEM cell p24 CA antigen production[c]
	Expt 1	Expt 2	Expt 1	Expt 2		
Mock	0	0	0	0	−	−
HIV-1 wild type	25	27	100	100	+	+
Δp2-composite	16	29	3	1	−	−
Δp2-slow	15	21	2	<1	−	−

[a] Percentage of medium-associated reverse transcriptase (RT) activity able to be pelleted by centrifugation through a 15% sucrose cushion. As a control, viral stocks of murine leukemia virus and avian leukosis virus were pelleted under the same conditions, with percentages of HIV-1 virion reverse transcriptase activity of 65 and 87%, respectively.
[b] The number of infectious particles by MAGI assay and pelletable reverse transcriptase activity.
[c] CEM cells were infected with 500 μl of medium collected 48 h posttransfection and monitored for cytopathic effect and p24 CA antigen production over a 3-week period.

ence in the rates of cleavage of the Gag sites in vitro. The role of the p2 domain as a negative regulator of the cleavage of the CA/p2 site in infected cells was also confirmed. This was evident by a significant increase in the formation of the MA/CA intermediate when the p2 domain was removed (Fig. 1, 4B, and 6).

It is not known whether a particular sequence or length of p2 is necessary for the regulation of cleavage at the CA/p2 site. p2 is poorly conserved in sequence between the primate lentiviruses (16) and can be variable in sequence and composition in different HIV-1 isolates (31). Cleavage sites embedded within the context of a large protein may simply be cleaved more efficiently by the HIV-1 PR than sites near the end of the protein precursor. We cannot exclude, however, that p2 may also perform a function other than that described here. Processing intermediates with small peptide domains may have a transient function during virion maturation. Such an example is seen with the bovine leukemia virus matrix protein, in which a carboxy-terminal extension is necessary for the binding of viral RNA (23). Nevertheless, p2 does function to regulate the rate at which the CA/p2 site is cleaved during sequential processing of HIV-1 Gag, and its presence is important for the production of fully infectious virus (Table 2). A report by Craven et al. (5) has also demonstrated the importance of the spacer peptide located between CA and NC of Rous sarcoma virus Gag to produce infectious virions.

The effect of pH on cleavage of the Gag sites. Processing reactions were performed at pH 5 to gain insights into the mechanism by which the p2 tail slows the rate of cleavage at the CA/p2 site. Lower pH makes a potential processing site sequence in native lactate dehydrogenase more accessible to cleavage by HIV-1 PR (54). Thus, processing reactions performed at lower pH may serve as a probe for conformationally sensitive areas. Varying the pH of in vitro assays has also been used as a probe for potential electrostatic interactions between proteinases and substrate (14, 18, 49). We found that the CA/p2 site was selectively accelerated by the drop from neutral pH relative to the other Gag sites. Accelerated cleavage at the CA/p2 site at lower pH occurred irrespective of the formation

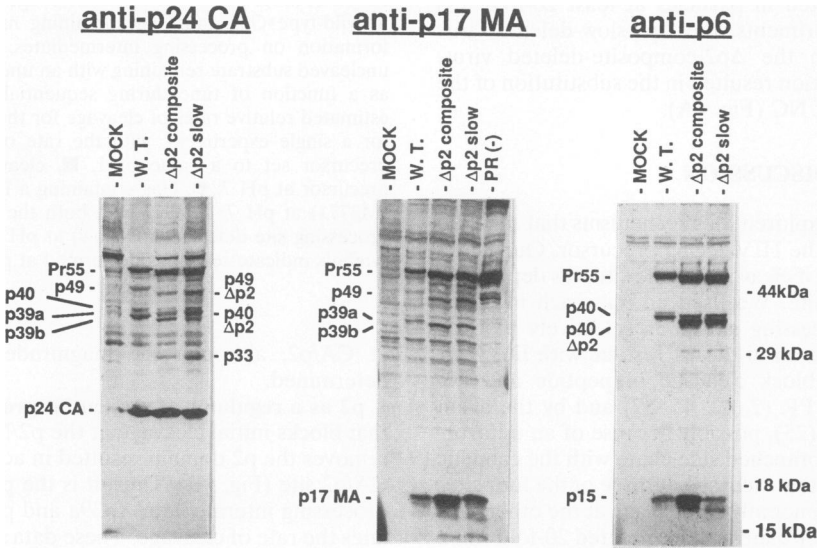

FIG. 6. Analysis of processing intermediates observed in cells transfected with the wild-type (W.T.) HIV-1 genome or genomes deleted in the p2 domain. Samples were analyzed by Western blotting with the indicated antibodies after SDS-PAGE on a 17% acrylamide gel. The positions of molecular mass markers are shown on the right. The positions of the Gag precursor, the composition of processing intermediates, and the final products are shown on the left of each panel. The deletion mutants used were Δp2-slow and Δp2-composite.

of the p2 tail (Fig. 5B), suggesting sequence-based electrostatic influences rather than changes in accessibility or conformation.

The influence of pH on the cleavage of the CA/p2 site was confirmed by the hydrolysis of a peptide substrate as well (Table 1). There is general agreement that the rate of hydrolysis of peptide substrates by HIV-1 PR increases at lower pH near pH 5 (3, 18, 19, 46). Previously published reports have noted that the sequence of the CA/p2 site is one of the best HIV-1 processing sites when assayed as a peptide at low pH (45, 55). Under conditions of lower pH and with a mutation preventing the formation of the p2 tail (M3771), we observed that the CA/p2 site in the native Gag precursor was cleaved faster than the MA/CA site (40). Evidently, in the virion, the combination of neutral pH and negative regulation by the p2 domain slows cleavage of the CA/p2 site so that it is one of the slowest Gag sites.

Dilanni et al. (7) and Richards et al. (45) also noted an increase in K_m for the hydrolysis of a peptide representative of the CA/p2 site at higher pH, although the exact magnitude of the increase at neutral pH was not determined. Our results suggest one or more of the residues in the P1′ to P3′ position of the CA/p2 site are responsible for the sensitivity to variations in pH. A P2′ Glu among the HIV-1 cleavage sites is unique to the CA/p2 site, and a P2′ Glu has been implicated in favorable recognition of heterologous cleavage sites in protein precursors (43, 52, 54), peptide substrates (15, 43), and substrate-based inhibitors by PR (32, 59). However, most of these studies have been based on experimental data collected near pH 5. Protonation of the P2′ Glu at lower pH may allow a more favorable association with the S2′ subsite of PR. In that case, a P2′ Glu in a substrate may be less favorable at physiological pH.

The influence of sequence and context on determining the rate of cleavage at a processing site. This report emphasizes the importance of context in regulating the rate of cleavage at a processing site. When the two sites flanking the p2 domain are placed in identical context in the native Gag precursor, the difference in the rates of cleavage was reduced from 400-fold to 15-fold (not shown). In addition, the change of context had a bidirectional effect on the rate of cleavage: the p2/NC site was decelerated and the CA/p2 site was accelerated. Tritch et al. (58) had previously noted the importance of context in influencing the rate when they observed that placement of the p2/NC cleavage site sequence at the MA/CA site resulted in cleavage that was an order of magnitude slower than the rate of cleavage in its normal context. The CA/p2 site of Gag may not be the only site influenced by dynamic changes in context or conformation. Sheng et al. have also noted that cleavage at the p1/NC site is influenced by the presence of RNA (50).

We also observed that processing sites differ in allowed amino acid substitutions. Leu substitutions reduced the rate of cleavage at the MA/CA junction more than the p2/NC site. This is in agreement with the suggestion of Partin et al. (38) that the MA/CA site may be more sensitive to mutation than other processing sites. The differential effects of the P1 Leu substitutions may reflect differences in amino acid preferences that have been noted for the aromatic/Pro and hydrophobic/hydrophobic classes of sites, of which these two sites are representative (15, 42).

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