JOURNAL OF VIROLOGY, Jan. 1994, p. 240–250
0022-538X/94/$04.00+0

Vol. 68, No. 1

Proteolytic Activity of Novel Human Immunodeficiency Virus Type 1 Proteinase Proteins from a Precursor with a Blocking Mutation at the N Terminus of the PR Domain

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Received 19 January 1993/Accepted 1 October 1993

The mature human immunodeficiency virus type 1 proteinase (PR; 11 kDa) can cleave all interdomain junctions in the Gag and Gag-Pol polyprotein precursors. To determine the activity of the enzyme in its precursor form, we blocked release of mature PR from a truncated Gag-Pol polyprotein by introducing mutations into the N-terminal Phe-Pro cleavage site of the PR domain. The mutant precursor autoprocessed efficiently upon expression in *Escherichia coli*. No detectable mature PR was released; however, several PR-related products ranging in size from ≈14 to 18 kDa accumulated. Products of the same size were generated when mutant precursors were digested with wild-type PR. Thus, PR can utilize cleavage sites in the region upstream of the PR domain, resulting in the formation of extended PR species. On the basis of active-site titration, the PR species generated from mutated precursor exhibited wild-type activity on peptide substrates. However, the proteolytic activity of these extended enzymes on polyprotein substrates provided exogenously was low when equimolar amounts of extended and wild-type PR proteins were compared. Mammalian cells expressing the mutated precursor produced predominantly precursor and considerably reduced amounts of mature products. Released particles consisted mostly of uncleaved or partially cleaved polyproteins. Our results suggest that precursor forms of PR can autoprocess but are less efficient in processing of the Gag precursor for formation of mature virus particles.

Human immunodeficiency virus type 1 (HIV-1) and other retroviruses express the structural and enzymatic functions required for assembly of infectious particles in two polyprotein precursors, Gag and Gag-Pol. In HIV-1, the synthesis of the Gag-Pol polyprotein requires translational frameshifting, which occurs with a frequency of about 5% of all translational events (12). The precursors are assembled at the cell membrane and released as immature viral particles which are converted into mature infectious virus through proteolytic processing by the virally encoded proteinase (PR) (15). The critical role of HIV PR in virus infectivity has made it a prime target for development of antiviral drugs and has resulted in a large data base on the structure and function of the enzyme (for reviews, see references 3, 6, and 36). Despite this progress, the mechanisms underlying the control of PR activity during intracellular synthesis and assembly of the polyprotein precursors and the activation of PR during or after particle release remain unknown.

The retroviral proteinases are aspartic proteinases (14, 28). Unlike the cellular members of this class of enzymes, which contain the catalytic apparatus in a single polypeptide, the viral enzymes must dimerize to be active (2, 23). Interdigitation of the N- and C-terminal regions of the mature viral PR monomers is critical for dimer stability (33, 40). Thus, it is anticipated that the proteolytic activity of PR-containing precursors in the cytoplasm would be limited by both concentration and structural constraints. This limitation of cytoplasmic processing is necessary to ensure efficient virus particle assembly, since premature precursor processing would result in separation of Gag and Pol domains from critical transport signals in the precursor. However, considerable amounts of processed Gag and Pol products can be detected in lytically infected cells, suggesting that precursors exhibit proteolytic activity which is not limited to extracellular particles (13, 22, 24).

While the PR-containing precursors of the avian and murine retroviruses have been found to be inactive in vitro or in *Escherichia coli*, HIV PR-containing precursors efficiently autoprocess in these systems. The HIV PR may be more active in vitro because (i) it is a catalytically more efficient enzyme, (ii) its activity is less regulated in vitro, or (iii) PR-containing precursors possess proteolytic activity. To investigate the proteolytic activity of the HIV-1 PR precursor and to determine whether inhibition of PR maturation affects autoprocessing, release of mature PR was blocked by mutation of the cleavage site at the N terminus of the PR domain. Precursors containing the mutation autoprocessed after translation in rabbit reticulocyte lysate (RRL) or when expressed in *E. coli*. Thus, blocking PR maturation did not inhibit autoprocessing, suggesting that HIV PR can be active in a polyprotein form. Examination of the PR species generated after autoprocessing in *E. coli* revealed several extended forms with N termini located in the p6* region immediately upstream of the PR domain. In this report, we describe the formation and activity of these extended species of HIV-1 PR and examine the role of PR maturation in production of mature particles.

MATERIALS AND METHODS

Plasmid constructions. The construction of the parent plasmids (pHIV-g/pII and pHIV-FSII) has been described previously (18). These plasmids contain a T7 promoter followed by HIV sequences from BH10 spanning nucleotides 221 to 2130

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(31). This region includes 113 nucleotides of the 5′ nontranslated region, the *gag* gene, that part of the *pol* gene that encodes PR, and stop codons. pHIV-FSII contains a 4-bp insertion at nucleotide 1640 of the HIV cDNA. In bacterial strains expressing the T7 RNA polymerase, g/pII and FSII allow synthesis of Gag/Gag-PR and Gag-PR, respectively, which are truncated forms of the Gag-Pol polyprotein. Plasmids for mutagenesis were constructed by transferring the *Eco*RI fragment from pHIV-g/pII or -FSII containing the Gag-PR sequence into pBS/KS (Stratagene). For expression in mammalian cells, the *Eco*RI fragment from plasmid pHIV-g/pII was subcloned into plasmid pBS-RRE and the *Sal*I-*Not*I fragment from the resulting plasmid, pBS-R-g/p, also containing the Rev responsive element, was inserted into pKEX-XR (25). This places the HIV coding sequence under control of the human cytomegalovirus promoter-enhancer and the simian virus 40 RNA processing signals. Plasmids were propagated in *E. coli* C600.

Site-directed mutagenesis. Mutagenesis was performed by the method of Kunkel, with minor modifications as previously described (19).

In vitro transcription and translation. DNA was linearized at the *Aat*II restriction site 3′ to the coding sequences, and runoff transcription reactions using 5 μg of linearized DNA per 100 μl and T7 polymerase were performed as previously described (18). Synthetic RNAs were translated in RRL (Promega), by the protocol of the supplier, in the presence of [^{35}S]methionine (specific activity, >1,000 Ci/mmol) (ICN). Translation products were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Expression in *E. coli*. Plasmid DNA was transformed into competent *E. coli* BL21(DE3). Bacteria carrying expression vectors were grown in minimal media (M9) as described before (17). Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After 45 min of induction, [^{35}S]methionine was added to a final concentration of 10 μCi/ml, and cells were labeled for 30 min.

Purification of proteinases. Extended forms of HIV PR expressed by pHIV-FS S/R in strain BL21(DE3) were partially purified by using protocols previously described for isolation of HIV PR and CA proteins (5, 17). Briefly, 10 g of bacterial cell paste (≈20% of the yield of cells grown in a 14-liter fermentor [Microferm MMF-14]) was resuspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) with 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA and lysed by using a French press (SLM Instruments). The bacterial lysate was clarified by centrifugation at 10,000 × *g* for 15 min, and the supernatant was further centrifuged for 60 min at 200,000 × *g*. Proteins from the final supernatant were precipitated with 30% ammonium sulfate. The precipitate was collected by centrifugation, redissolved in 50 mM Tris-HCl buffer (pH 8) containing 30 mM NaCl and 1 mM EDTA, and chromatographed on a Whatman DE52 DEAE-cellulose column which had been equilibrated with the same buffer. Unbound proteins in the flowthrough fraction were precipitated by the addition of ammonium sulfate to 50% (wt/vol) and resuspended in 50 mM Tris-HCl buffer (pH 8) containing 30 mM NaCl and 1 mM EDTA to a protein concentration of about 30 mg/ml (≈1-ml volume). After incubation at 4°C overnight, the solution was clarified by centrifugation, and the supernatant fraction was assayed for proteolytic activity. The extended PR forms accounted for approximately 0.1 to 0.4% of the total protein in this fraction, as estimated by Coomassie blue staining of the bands in SDS-polyacrylamide gels.

Protein analyses. Concentrations of protein samples were measured with the Bio-Rad protein-dye binding assay. Proteins were electrophoresed under denaturing conditions with Laemmli gels (20). For analysis of the extended PR forms, these conditions were modified by increasing the final concentration of Tris-HCl (pH 8.8) from 0.375 to 0.75 M. Protein bands were visualized by staining with Coomassie blue or by immunological detection. Immunological analysis of expressed proteins was done either by precipitation with specific antibodies followed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography or by immunoblotting onto nitrocellulose filters by a procedure essentially as described by Towbin et al. (37). Primary polyclonal antisera were purchased from DuPont Co. or raised in rabbits against purified CA protein (5) or against PR. Monoclonal mouse antibodies directed against PR were also used. Anti-PR antibodies are generous gifts of P. Barr, Chiron Corporation, and M. C. Graves, Department of Molecular Genetics, Hoffman-La Roche, Inc. Secondary antibodies were either alkaline phosphatase conjugated and used for color-developed blots or conjugated with horseradish peroxidase and used on blots developed by enhanced chemiluminescence (ECL; Amersham). Alkaline phosphatase-conjugated antibodies were goat anti-rabbit immunoglobulin (IgG) from Tago, Inc., and from Dianova, Hamburg, Germany. Horseradish peroxidase-conjugated antibodies were donkey anti-rabbit IgG and sheep anti-mouse IgG from Amersham. Automated Edman degradation of electroblotted proteins was performed by using a gas-phase protein sequencer model 470A equipped with a PTH analyzer model 120A (Applied Biosystems) with standard programs recommended by the manufacturer.

Peptide cleavage. (i) Analysis by HPLC. The decapeptide RRELQVWGRD (synthesized by Multiple Peptides Systems) was checked for purity by high-pressure liquid chromatography (HPLC). For estimation of kinetic parameters, enzyme activity on the substrate was assayed in 50 mM MES (pH 5.0)–1 M NaCl at 30°C. The substrate concentration (quantitated by amino acid analysis) was varied from 0.25 to 2 mM. The concentration of highly purified HIV-1 PR (a generous gift of M. C. Graves, Department of Molecular Genetics, Hoffman-La Roche, Inc.) (9) was determined by active-site titration to be 200 nM. The reaction was stopped before ≈30% of the substrate was cleaved. During this period (<4 h for RRELQVWGRD), the reaction was linear with time. After incubation of peptide with PR, the reaction products were separated on an ABI 300 reverse-phase Aquapore C8 analytical column. The hydrolyzed product was analyzed by A_{220}. The hexapeptide QVWGRD was employed as a standard. Enzyme hydrolysis rates were fit to the Michaelis-Menten equation by using a nonlinear regression program (Enzfitter, Biosoft).

(ii) Spectrophotometric analysis. The peptide substrate K-A-R-I-Nle-Nph-E-A-Nle-NH_2 was synthesized and purified by HPLC as described previously (32). The buffer used was 0.1 M sodium acetate (pH 4.7) containing 4 mM EDTA and 1 M NaCl. The active concentrations of PR preparations were determined by active-site titration (4). Assays for hydrolysis catalyzed by partially purified wild-type PR and extended PR forms utilized volumes of ≈200 μl, corresponding to an active concentration of ≈20 nM. Initial velocities were measured for several substrate concentrations, and the kinetic parameters were derived from a computer fit of the data by using the Enzfitter program.

Proteolytic processing of polyproteins (in *trans*). To determine the activity of wild-type and extended PR forms, bacterial cultures expressing parent and mutated plasmids were lysed by sonication in PR cleavage buffer (50 mM MES [pH 6], 20 mM NaCl, 5 mM EDTA). Increasing volumes (1 to 12.5 μl) of

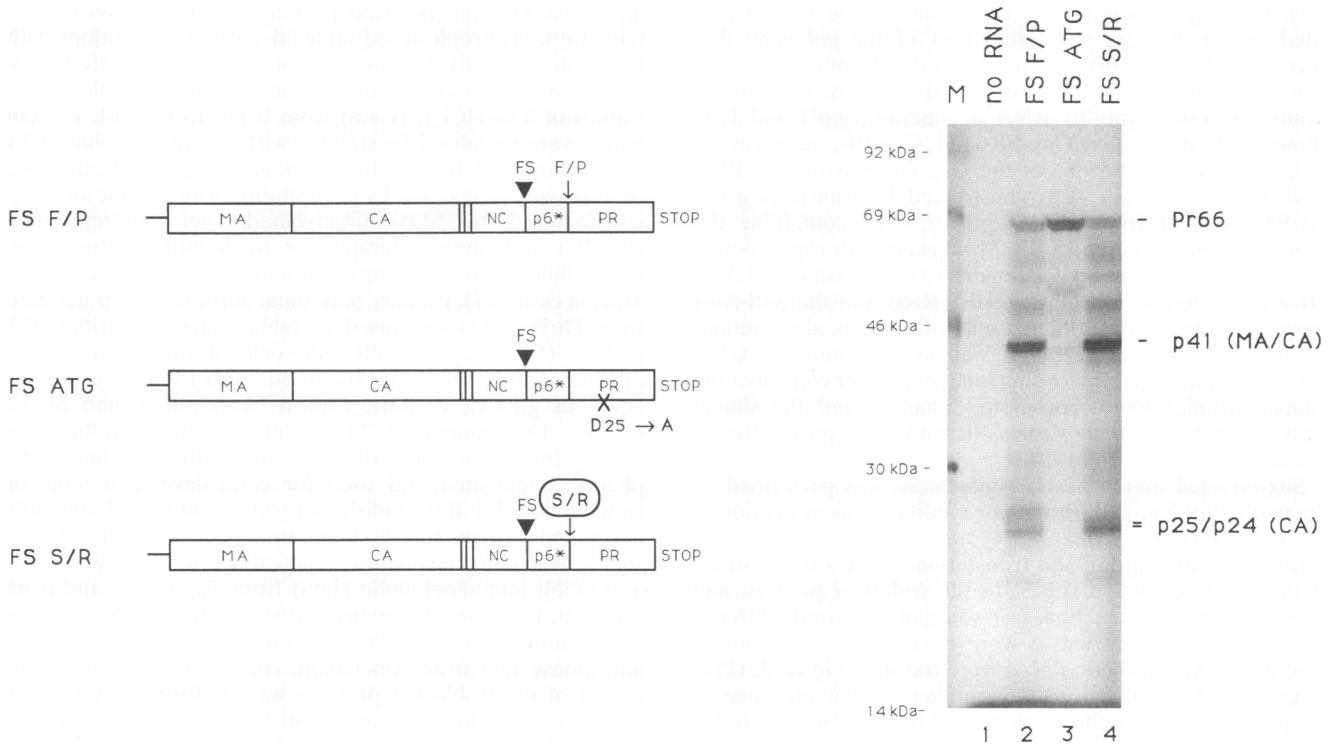

FIG. 1. Construction and in vitro translation of vectors permitting equimolar expression of wild-type PR (FS F/P), catalytically inactive PR (FS ATG), and N-terminally blocked PR (FS S/R). (Left) Open boxes represent proteins encoded by the HIV-1 sequences from nucleotide 333 to 2130 of BH10 (31). The nontranslated region (nucleotide 221 to 332) is indicated by a line. The products of the *gag* gene include MA, CA, and NC. The products of the *pol* gene are p6* and PR. The position of a 4-bp insertion that results in translational reading of *pol* sequences in the *gag* reading frame is indicated as an inverted closed triangle. (Right) Autoradiograph of an SDS–12.5% PAGE analysis showing the major gene products detected after in vitro translation of synthetic RNAs in RRL in the presence of [^{35}S]methionine. Lane 1, no RNA added; lane 2, FS F/P; lane 3, FS ATG; lane 4, FS S/R. Pr66 represents the full-length precursor polyprotein, p41 (MA/CA) is the MA-CA processing intermediate, and p25/p24 (CA) represents the two CA species with different C termini.

wild-type and mutant lysates were incubated with substrate in a final volume of 25 μl and in the presence of RNase (20 μg/ml) and cycloheximide (0.6 mM). The substrate was radiolabeled FS ATG precursor synthesized in RRL. FS ATG encodes an inactivating mutation in the catalytic site of PR (26). Reaction mixtures were incubated at 30°C for 1 h, and reactions were stopped by addition of SDS-PAGE sample buffer and boiling. Cleavage products were analyzed by SDS-PAGE as described above. The amounts of CA and PR proteins present in the bacterial lysates were determined by immunodetection by using ECL and quantitated by scanning densitometry with a LKB Ultroscan XL laser densitometer.

Expression in mammalian cells. COS 7 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Transient expression experiments were performed essentially as previously described (25). Briefly, $\approx 5 \times 10^6$ cells were suspended in 0.1 ml of phosphate-buffered saline and electrotransfected by using a Bio-Rad gene pulser equipped with a capacitance extender and pulse controller. Transfected cells were diluted into 20 ml of warm fresh medium, plated on two 10-cm-diameter dishes, and incubated for 48 h after transfection. Cells were harvested by being scraped off the plate and lysed in 2% SDS. For detection of HIV antigens, media were cleared and appropriate dilutions were analyzed by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Organon Teknika, Eppelheim, Germany) detecting primarily the cleaved CA protein. Dilutions were chosen to be in the linear response range of the ELISA. Virus-like particles were obtained by sedimentation of cleared media (200 × *g*, 10 min) through a cushion of 20% (wt/vol) sucrose at 120,000 × *g* for 2 h at 4°C.

RESULTS

Blocking cleavage at the N terminus of HIV-1 PR does not prevent precursor processing. The expression vectors used for these experiments consisted of plasmids encoding all of *gag* plus that part of the *pol* gene encoding PR, inserted downstream of a promoter for T7 RNA polymerase (Fig. 1, left). Construct gp, which we have previously described (18), expresses predominantly Gag and relies on ribosomal frameshifting for expression of polyproteins containing PR (see below). Since this event is infrequent, processing of the precursor occurs only to a limited extent. To express only the precursor containing PR, we introduced a forced frameshift by inserting 4 bp very close to the natural frameshift site. This construct, called FS, autoprocesses all sites of the precursor with high efficiency (18). Constructs FS and gp were used as the parents for site-directed mutagenesis in this study.

The high proteolytic activity exhibited by FS could be attributed to the release of mature PR by autoprocessing, or it might reflect the activity of precursors and cleavage intermediates. To determine the proteolytic activity of PR-containing polyproteins, the Phe-Pro cleavage site at the N terminus of

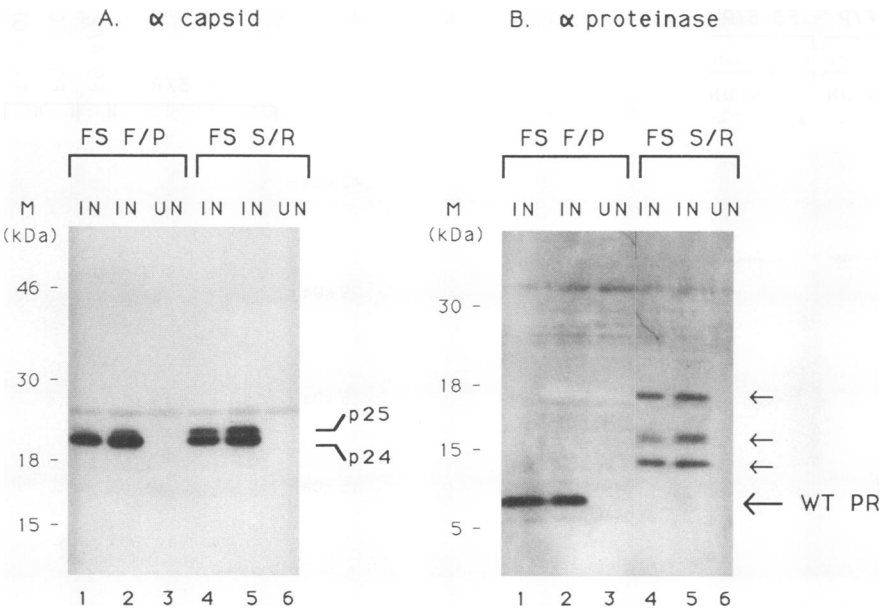

FIG. 2. Expression of constructs FS F/P and FS S/R in *E. coli* BL21(DE3). Transformed bacteria were induced with 0.4 mM IPTG. The proteins in total cell lysates were separated by SDS-PAGE (10 to 20% gradient gel) and analyzed by immunoblotting with rabbit antisera directed against CA protein (A) and mouse monoclonal antibody directed against PR (B). Immunodetection was performed by ECL. Lanes 1 and 2, induced lysates of bacteria transformed with FS F/P; lanes 4 and 5, induced lysates of bacteria transformed with FS S/R; lanes 3 and 6, uninduced lysates. Lanes 2 and 5 contain twice the amount of lysate loaded in lanes 1 and 4, respectively. (B) The three upper arrows indicate the positions of the major PR products detected in lysates of cells carrying FS S/R. The larger arrow below indicates the position of the mature, 11-kDa wild-type (WT) PR.

the PR domain in our Gag-PR precursor was changed to Ser-Arg (Fig. 1, left). This dipeptide was chosen because polar and charged amino acids have not been observed in retroviral PR cleavage sites (29). In addition, Göttlinger et al. have shown that mutation of the MA-CA and CA-p2 junctions to S-R completely blocked cleavage at these sites (8). Parent (FS F/P) and mutated (FS S/R) constructs were transcribed in vitro by T7 RNA polymerase, and equimolar amounts of the synthetic RNAs (70 nM) were translated in RRL in the presence of [^{35}S]methionine. SDS-PAGE analysis of the translation products (Fig. 1, right) revealed little difference in autoprocessing of the mutated (lane 4, FS S/R) and parent precursors (lane 2, FS F/P). Both were autoprocessed into p41, a MA-CA intermediate, and into mature p24 capsid protein. The protein detected in lane 2 migrating slightly slower than p24 and p25 is a PR-containing intermediate and can be precipitated with antiserum directed against PR (26). p25 (lane 3) consists of the CA domain plus the p2 domain at the C terminus of CA. The mature MA protein contains no methionines and cannot be detected by labeling with [^{35}S]methionine. NC (7 kDa) and PR (11 kDa) were not retained under these conditions of gel electrophoresis. Thus, the presence of p41 and p24 products indicated that the mutated precursor retained the ability to catalyze autoprocessing. Detection of p25 in mutant but not wild-type lysates suggested that processing by the mutant was slightly less efficient. The results also indicate that mutation of the first amino acid of the PR domain, Pro to Arg, did not significantly impair the autoprocessing activity of the enzyme. Control construct FS ATG (lane 3) contained a mutation in the active site of PR, Asp-25 to Ala, and did not give rise to any processed products.

To obtain sufficient amounts of proteins for further analysis, the mutated construct was expressed in *E. coli*, in which we had previously demonstrated rapid and complete wild-type polyprotein processing with accumulation of mature PR (5). Crude lysates from uninduced and induced bacteria transformed with the parent (FS F/P) or the mutated (FS S/R) construct were examined by Western blotting (immunoblotting) with polyclonal antisera directed against the capsid protein, CA (p24) (Fig. 2A). Both parent and mutated precursors autoprocessed very efficiently, since no CA-containing precursors were detected. The parent construct, FS F/P (lanes 1 and 2), gave rise to fully processed p24 and only traces of p25. In contrast, both p24 and p25 were present in near equal amounts in the lysate of cells expressing the mutated construct FS S/R (lanes 4 and 5), indicating slightly less efficient cleavage. After longer induction periods, no p25 was detected and comparable levels of p24 were present in both lysates. Thus, although both wild-type and mutated precursors appear to undergo efficient autoprocessing in vitro and in *E. coli*, processing by the mutant is slightly less efficient in both systems.

To determine the form of PR that accumulated, the same crude bacterial lysates were analyzed by Western blotting for products reactive with anti-PR antibody (Fig. 2B). As expected, bacteria carrying the parent construct expressed PR as a mature 11-kDa protein (lanes 1 and 2, WT PR). In contrast, lysates of bacteria expressing the mutated construct contained several anti-PR-reactive proteins (lanes 4 and 5). Three major bands that ranged in size between 14 and 18 kDa were detected. The same relative amounts of products were detected by using three different antibodies directed against PR and in Coomassie blue-stained gels (data not shown). These results indicated that alternative cleavage sites were used to release PR from the precursor.

Specificity of the autoprocessing reaction. To confirm that the 14- to 18-kDa PR species were generated by HIV-1 PR and not by enzymes present in *E. coli*, we used radiolabeled precursors synthesized in RRL as substrates for purified wild-type 11-kDa PR. The cleavage products were immunoprecipitated with polyclonal antibody directed against PR, and

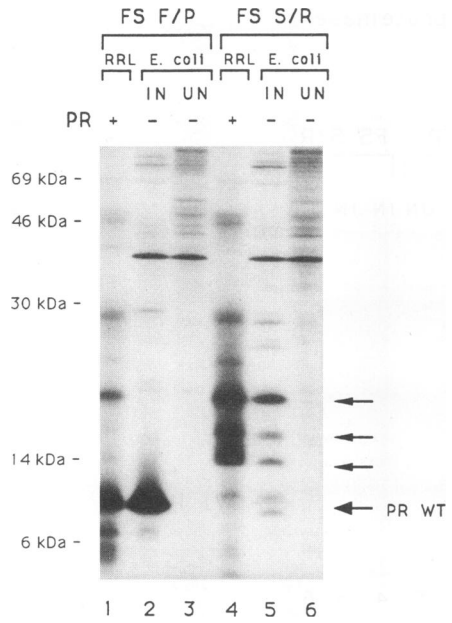

FIG. 3. Comparison of PR species expressed in RRL and *E. coli*. FS F/P and FS S/R were translated in RRL in the presence of [^{35}S]methionine (cf. Fig. 1). In vitro translation products were incubated with a 50-fold excess of purified, 11-kDa mature PR (9) followed by immunoprecipitation with rabbit antisera directed against PR. The samples were analyzed in parallel with radiolabeled PR species immunoprecipitated from *E. coli* lysates on 10 to 20% SDS-polyacrylamide gradient gels and by autoradiography. Lanes 1 to 3, PR species from FS F/P generated in RRL (lane 1), induced *E. coli* (lane 2), and uninduced *E. coli* (lane 3); lanes 4 to 6, PR species from FS S/R generated in RRL (lane 4), induced *E. coli* (lane 5), and uninduced *E. coli* (lane 6). Arrows indicate major PR species in FS S/R and wild-type (WT) PR in FS F/P.

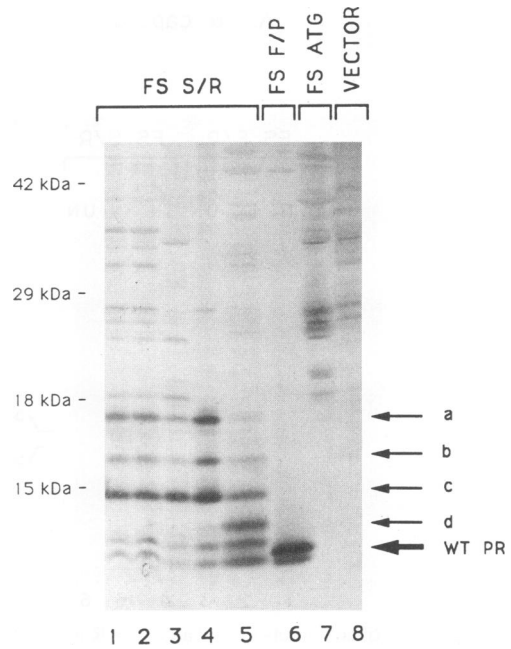

FIG. 4. Fractionation of total lysate from induced bacteria transformed with FS S/R and control constructs. Induced cells were lysed and fractionated as described in Materials and Methods. Samples of fractions after each step of purification containing ≈40 μg of total protein were separated by SDS-PAGE (10 to 20% gradient gel) and analyzed by immunoblotting with rabbit antisera directed against PR and secondary antibody conjugated with alkaline phosphatase. Lane 1, total lysate after French press; lane 2, S10 supernatant; lane 3, S200 supernatant; lane 4, 30% ammonium sulfate precipitate; lane 5, 50% ammonium sulfate precipitate of flowthrough after DEAE-cellulose chromatography and after concentration of the sample to ≈30 mg of protein per ml. Lane 6 is equivalent to lane 5, except PR was generated from induced cells transformed with FS F/P. Lane 7, crude lysate of induced cells transformed with FS ATG. Lane 8, crude lysate of induced cells transformed with vector alone. PR species a to d are indicated by arrows. Boldface arrow indicates 11-kDa mature PR (WT PR).

the products detected were compared with those found in bacteria transformed with the parent or mutated constructs. The results are shown in Fig. 3. The major PR-related product obtained by cleavage of the parent precursor, FS F/P, expressed in RRL was the 11-kDa form of PR (Fig. 3, lane 1, WT PR). This is the same size PR species that was precipitated from lysates of induced (lane 2) but not uninduced (lane 3) bacterial cells transformed with the parent construct. In contrast, three major and several minor PR-related products were generated by cleavage of the mutated precursor, FS S/R (lane 4). The three major products comigrated with the PR species that accumulated in lysates of induced (lane 5) but not uninduced (lane 6) bacteria transformed with the mutated construct. Some of the minor proteins comigrated with minor products of autoprocessing that were observed infrequently in crude lysates but accumulated during partial purification (see Fig. 4). This experiment was performed several times with HIV-1 PRs generated in different strains of *E. coli* and purified by different methods (5, 9, 18), making it unlikely that a copurifying *E. coli* protease was responsible for the cleavage events. Moreover, no proteolytic activity was detected in purification fractions from uninduced cells or from induced cells transformed with vector alone (data not shown).

The products generated by cleavage with exogenously added wild-type PR appeared identical to those produced by autoprocessing. Thus, the extended forms of PR that accumulated by autoprocessing when release of the mature enzyme was blocked arise from the specific action of the virus-encoded proteinase. Moreover, we noted that small amounts of similar-size products also were produced from the parent precursor (Fig. 3, lane 1), suggesting that such PR forms could be transient cleavage products in the pathway leading to PR release. While dissimilar PR products were obtained from parent and mutated precursors, the precursors were cleaved with equal efficiency into identical Gag-related products. Thus, the mutation did not alter the overall conformation of the precursor.

The presence of p6* sequences in the extended PR forms was established by Western blotting with antibody raised against part of this region (data not shown). To determine where cleavage occurred in p6*, the PR species were partially purified from a large-scale culture of *E. coli* and analyzed by N-terminal sequencing. For comparison, wild-type PR was purified in parallel experiments. The expected 11-kDa mature PR and a smaller product arising from autolysis were obtained. The PR species expressed from the mutated construct (Fig. 4, lanes 1 to 5) were found in the soluble fractions and purified like wild-type PR (lane 6). Examination of the pellet fractions did not reveal any additional higher-molecular-weight forms of PR. While the 14- to 18-kDa species were stable throughout most of the purification procedure, they were unexpectedly found as lower-molecular-weight species after the last step of

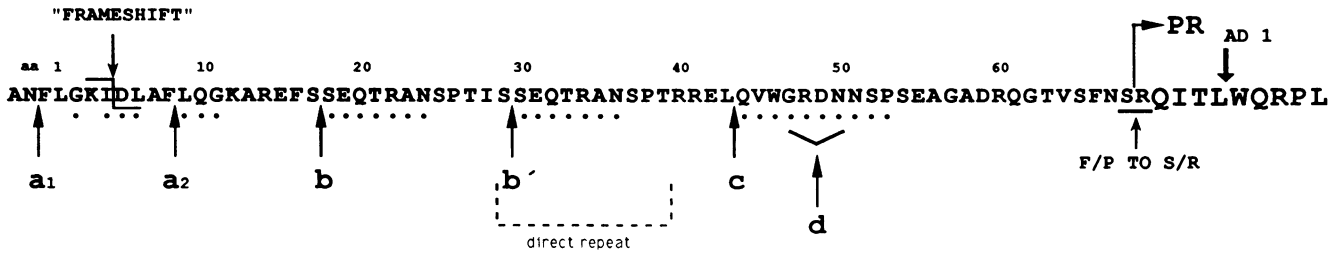

FIG. 5. Amino acid sequence of the p6* region of HIV-1 BH10 (31). The positions of the insertion in FS resulting in forced frameshifting, and the F/P to S/R mutation at the N terminus of the PR domain are included. The arrows underneath the sequence indicate the N termini of PR species a to d from Fig. 4. Residues identified by N-terminal analysis are marked by dots under the sequence. a_1 and a_2 indicate residues detected in the doublet in band a in figure 4. b and b′ indicate residues in a sequence that is duplicated in BH10. Assignments for b, c, and d are based on at least two independent analyses. Also shown is the position of the autodegradation site within the PR domain (AD 1) (35).

purification (lane 5). As previously described (5), this last step of purification involves precipitation of total protein by addition of $(NH_4)_2SO_4$ followed by resuspension to a final protein concentration of ≈30 mg of protein per ml and incubation at 4°C overnight. The instability of the 14- to 18-kDa forms under these conditions suggests that these PR species might serve as substrates for cleavage when protein-protein interaction is facilitated at a high PR concentration. In control experiments, no proteins of similar size were detected in supernatant (lane 7 and 8) or pellet (not shown) fractions derived from bacteria expressing precursors with inactive PR (lane 7, FS ATG) or from bacteria transformed with vector alone (lane 8). The possibility that the mutation (F-P to S-R) distorted the precursor in such a way that it became a target for bacterial proteases which did not attack the FS ATG precursor cannot be completely excluded. However, the observation that both the mutated and the wild-type precursors were cleaved equally well by wild-type PR (Fig. 3) makes it highly unlikely that such conformational distortions of the mutated precursor occurred.

The N-terminal sequences of the PR forms designated PR a to d in Fig. 4 are indicated in Fig. 5, which shows the amino acid sequence of p6* (BH10 [31]), the region upstream of PR. Species a was a doublet of very closely migrating proteins and contained fragments derived by cleavage near the N terminus of the p6* domain (a_1 and a_2). One of these sites (a_1) has been described (11). The other site (a_2) has also been observed recently (10a, 30). Species b and c were derived by cleavage within the p6* domain. The sequence at the N terminus of species b is redundant in clone pHIV-BH10, and thus the site of cleavage could not be mapped precisely. Species d was not always observed, but its N terminus was mapped repeatedly to amino acids 48 to 50 of p6*. The N terminus of a fifth PR species migrating faster than species d but more slowly than wild-type PR appears to be located at or near amino acid 60 of p6* (not indicated). None of these sites has been previously described. The smallest PR species (Fig. 4, lane 5) comigrated with the autodegradation product of wild-type PR (Fig. 4, lane 6, and Fig. 5, AD 1) (35). This product lacks the N-terminal region of PR which contributes to the stability of the dimeric structure.

From these newly described cleavage sites, the amino acids flanking the site yielding product c (Fig. 5) were selected as a representative substrate to verify that there is an authentic cleavage site for HIV-1 PR in the p6* domain. A synthetic decapeptide containing the sequence R-R-E-L-Q-V-W-G-R-D was incubated with purified, wild-type mature PR. The results are shown in Table 1. The peptide was cleaved specifically at Leu-Gln. A product corresponding to Q-V-W-G-R-D was recovered as judged by comparison with the elution position of purified synthetic Q-V-W-G-R-D. The other expected product, R-R-E-L, is hydrophilic and was not retained on the column. Initial rate data obtained by using several substrate concentrations in buffer containing 1 M NaCl (pH 5.0) at 30°C yielded values for kinetic parameters as follows: K_m = 1.3 mM and k_{cat} = 0.013 min^{-1} (Table 1). For comparison, cleavage of a known substrate, VSQNY/PIVQN containing the site at the amino terminus of the CA domain in Gag, was examined in parallel experiments. Under the conditions of the assay, a K_m value of 0.35 mM was determined for cleavage of the Y-P bond. This value is in agreement with previously described values (38). Comparison of the relative efficiencies of hydrolysis, k_{cat}/K_m (Table 1), indicated that cleavage at L-Q occurred very slowly. These experiments demonstrate that the L-Q site in the p6* domain is cleaved in the context of a polyprotein as well as in a peptide and thus represents an authentic cleavage site for HIV-1 PR.

Proteolytic activity of the extended proteinases. (i) Activity on peptides. The ability of the PR species that accumulated in bacterial cells expressing construct FS S/R to cleave peptide substrates was compared with that of the 11-kDa wild-type enzyme. A partially purified fraction containing all of the extended PR forms (Fig. 4, lane 5) was used as source of enzyme. The active concentrations of this preparation and that of the wild-type enzyme were determined by active-site titration (4). A highly sensitive, soluble chromogenic peptide, K-A-R-I-Nle-Nph-E-A-Nle-NH_2 (32) was used as a substrate. Initial rate data obtained by using several substrate concentrations in buffer containing 1 M NaCl (pH 5.0) yielded values for kinetic parameters as follows: K_m = 25 μM and k_{cat} = 23 s^{-1}. Similar values were obtained for wild-type PR (K_m = 35 μM, and k_{cat} = 35 s^{-1}) (Table 2). Western blot analysis indicated that the composition of the enzyme preparations remained unchanged during the course of the assay (data not shown). The total amount of smaller PR species migrating near 11 kDa in the preparation of FS S/R products (ahead of species d in Fig. 4) was insufficient to account for the observed amount of peptide hydrolysis. This conclusion is based on the observation

TABLE 1. Kinetic measurements with peptide substrates VSQNY/PIVQN and RREL/QVWGRD[a]

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
VSQNY/PIVQN	0.35	0.230	6.6×10^{-1}
RREL/QVWGRD	1.32	0.013	0.1×10^{-1}

[a] Assays were performed, as described in Materials and Methods, in 50 mM MES (pH 5.0) and 1 M NaCl.

TABLE 2. Kinetic parameters for hydrolysis of a peptide substrate by wild-type and extended PR forms[a]

PR	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
FS F/P	35	35	1.0
FS S/R	25	23	1.1

[a] Assays were performed, as described in Materials and Methods, in a final volume of 200 μl in 0.1 M sodium acetate (pH 4.7) containing 4 mM EDTA and 1 M NaCl.

that an amount of wild-type PR protein equivalent to that of the smaller PR species hydrolyzed far less peptide in control experiments. This conclusion is also based on the assumption that the proteolytic activity of these proteins migrating close to 11 kDa in the preparation of FS S/R products would be similar to that of mature (11-kDa) PR. Thus, the 14- to 18-kDa PR species must be contributing to the observed activity.

Since this experiment analyzes only the combined activity of all PR species, it will be necessary to purify each extended PR species separately for determination of their relative activity. Nevertheless, the results suggest that the N-terminally extended 14- to 18-kDa PR species are efficient enzymes on exogenous peptide substrates. A further implication of this observation is that substitution of Arg for Pro at the N terminus of PR does not detectably impair the activity of the enzyme.

(ii) Activity on polyprotein substrates. To assay for differences in activity by using a polyprotein substrate, comparable amounts of wild-type 11-kDa PR and the mixture of extended PRs were tested on Gag-PR substrate provided exogenously (Fig. 6). Western blots immunodetected with sensitive ECL were used to identify the major PR-related proteins in crude bacterial lysates. Quantitation of PR by densitometry of the resulting autoradiographs permitted determination of the total amount of PR protein in the lysates (cf. Fig. 2B and 6). The substrate (full-length and internally initiated polyproteins synthesized from construct FS ATG in RRL) was completely stable to incubation in buffer alone (Fig. 6A, lane 7). The wild-type PR present in the FS F/P lysate was more active in cleavage (lanes 1 to 6) than comparable amounts of all extended PR-species present in FS S/R lysates (lanes 8 to 13). As shown in Fig. 6B and C, 5 U of the extended PR species was required to obtain the levels of CA product generated by 1 U of the wild-type PR. The PR composition of the lysates remained unchanged during the course of the assay, which did not exceed 1 h of incubation (data not shown). The results suggest that when total amounts of PR proteins are compared, the combined activity of the N-terminally extended PRs on polyprotein substrates is lower than that of wild-type PR despite the fact that equivalent amounts of the dimeric forms as determined by active-site titration were equally efficient (Table 2). Thus, maturation of PR from the Gag-Pol precursor may be critical for efficient dimerization and for processing of Gag polyproteins. Alternatively, the extensions on PR could make the active site less accessible for polyprotein precursors.

The observed reduced level of activity of the mutated forms of PR on exogenously added Gag substrates suggested that release of mature PR may be critical for efficient processing of Gag. This possibility was examined by substituting the Ser-Arg mutation for Phe-Pro at the natural cleavage site in constructs that required ribosomal frameshifting for PR expression (Fig. 7, left). Figure 7 (right) shows the results obtained from transformed bacterial cells analyzed for CA protein expression by Western blotting. Relative to construct FS F/P (Fig. 2, lanes 1 and 2), construct gp F/P produced slightly less p24 and more p25 (Fig. 7, lanes 1 and 2). This result is expected, since this construct expressed levels of the 11-kDa mature PR that were ≈10- to 20-fold less than the level that accumulated when frameshifting was not required (data not shown). The efficiency of ribosomal frameshifting in *E. coli* is ≈5% (4b). Processing by the construct that both encoded the mutation and required ribosomal frameshifting for PR expression (gp S/R) was impaired relative to that of the parent construct gp F/P. Compared with the parent, the mutant accumulated little or no mature capsid protein (p24) and increased levels of intermediates that migrated at 25, ≈28, and 41 kDa (Fig. 7, lanes 4 and 5). The 41-kDa protein was identified as an MA-CA intermediate (data not shown). The identity of the ≈28-kDa protein is unknown. In Fig. 7, lane 5, which contained twice the amount of lysate compared with that in lane 4, intermediates larger than 41 kDa including residual $Pr55^{gag}$ precursor were detected. The species between 55 and 41 kDa lacked the C-terminal domain of the Gag precursor (4a). PR in cells induced to express gp S/R was below detectable levels. Assuming that the frameshift efficiency for mutant and wild-type construct was equal, our failure to detect PR in gp S/R lysates suggests that several PR species were produced and that each one was below the limit of detection. Since substitution of Arg for Pro at the N terminus of PR did not detectably impair the activity of the enzyme, the results suggest that blocking PR N-terminal cleavage produces less-efficient extended forms of the enzyme.

Effect of cleavage site mutation on polyprotein processing and particle formation in eukaryotic cells. To determine whether release of mature PR was an important factor for polyprotein processing and particle assembly in eukaryotic cells, the construct encoding the mutated polyprotein and requiring frameshifting for PR expression (gp S/R) was adapted for expression in mammalian cells. It had been demonstrated previously that coexpression of *gag*, *pol*, and *rev* genes by transient transfection resulted in synthesis and proteolytic processing of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ and the assembly of virus-like particles (25, 34). Extracts prepared from transfected COS 7 cells and particulate material precipitated from the media were analyzed by Western blotting with polyclonal antiserum against CA protein (Fig. 8). No signal was detected in samples derived from cells transfected in the absence of Rev (data not shown). Extracts prepared from cells transfected with the wild-type plasmid, gp F/P, contained $Pr55^{gag}$, the p41 MA-CA intermediate, and CA proteins p24 and p25 (Fig. 8A, lane 1). Intracellular processing has been reported previously (13). As predicted from the results above (Fig. 6 and 7), mutation of the scissile bond at the N terminus of the PR domain reduced the efficiency of processing but did not block cleavage of the precursor, as evident by the accumulation of the p41 intermediate (Fig. 8, lane 2). Results consistent with those described were obtained by using identical blots probed with serum from an HIV-1-infected individual or with antibodies against MA or NC (p15) (data not shown).

The tissue culture media from transfected cells were examined for Gag-related particulate products (Fig. 8B). Particles released from cells transfected with the wild-type construct gp F/P contained mainly mature p24 and smaller amounts of $Pr55^{gag}$ and p41 (Fig. 8B, lane 1). CA protein migrated as a single band, as is characteristic of the mature protein in infectious particles. The presence of virus-specific proteins in the media indicated that the F-P to S-R mutation had no detectable effect on particle release; however, processing of the precursors to mature CA within the particle was significantly reduced (Fig. 8B, lane 2). Extracellular particles consisted

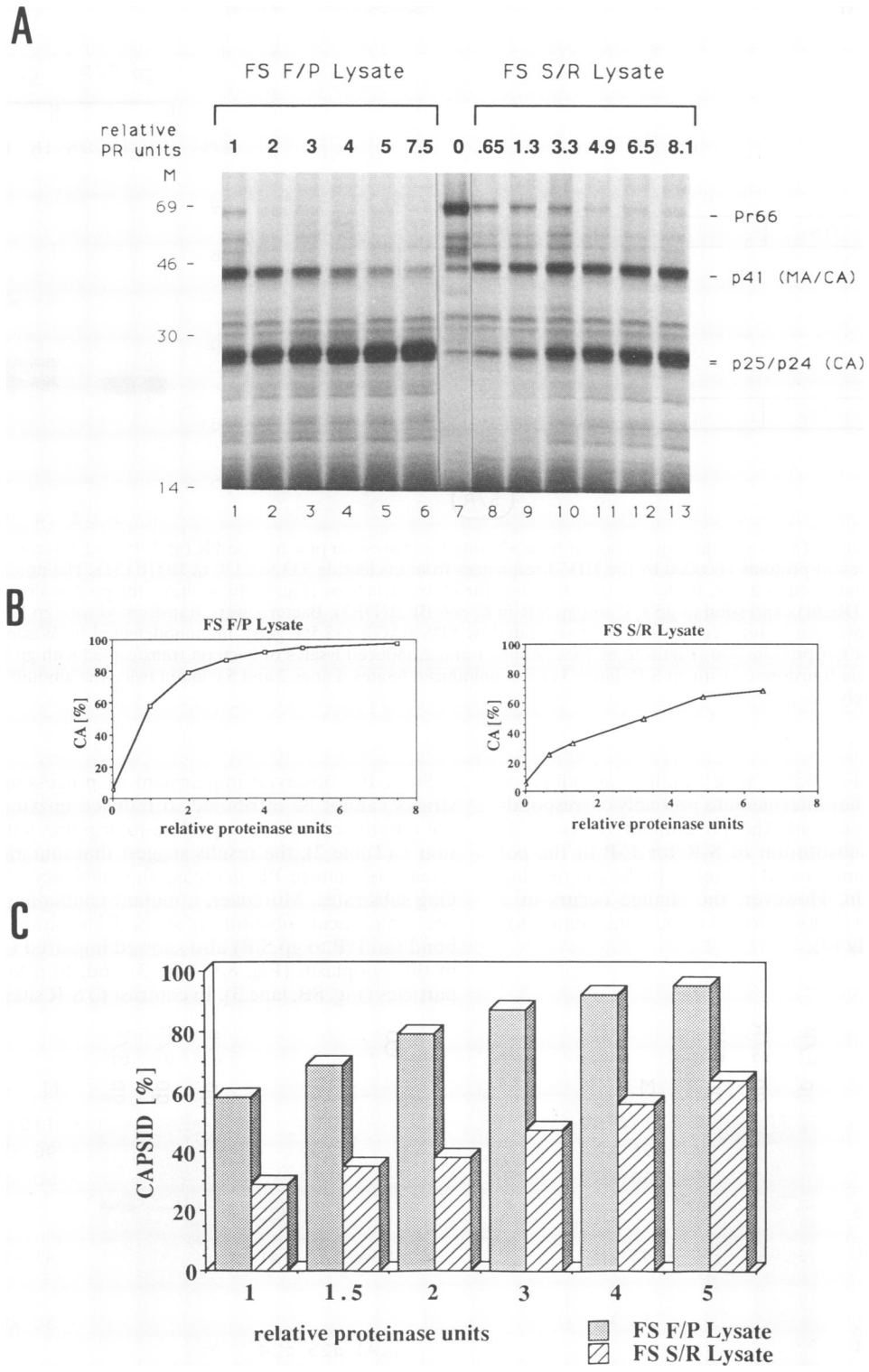

FIG. 6. Proteolytic processing of polyprotein substrates. Synthetic FS ATG RNA encoding a proteolytically inactive truncated Gag-Pol precursor (Pr66) was translated in RRL in the presence of [^{35}S]methionine and served as substrate. For the cleavage assay, 2 μl of the translation reaction was incubated with buffer alone (lane 7) or with increasing volumes of crude lysates of bacteria expressing construct FS F/P (lanes 1 to 6, 1 to 5 and 7.5 μl, respectively) or construct FS S/R (lanes 8 to 13; 1, 2, 5, 7.5, 10, and 12.5 μl, respectively). The cleavage reactions were analyzed by SDS-PAGE on 12.5% gels followed by autoradiography. PR-related proteins in these lysates were immunodetected by ECL and quantitated by scanning densitometry. The amount of PR species detected in FS S/R lysate equaled 65% or more of the amount of 11-kDa PR present in FS F/P lysate (data not shown). Relative PR units were then defined by assuming that the amount of PR present in 1 μl of FS F/P lysate equals 1 U while the amount of PR proteins in FS S/R lysate equals 0.65 U/μl. (B) Efficiency of cleavage was measured by scanning densitometry of all lanes in panel A and the amount of p25/p24 capsid protein generated by cleavage was expressed as a percentage of all radiolabeled proteins present in the respective lane. The percentage of CA was plotted against relative PR units. (C) Comparison of cleavage efficiency of the two lysates. The CA values were derived from the plot shown in panel B.

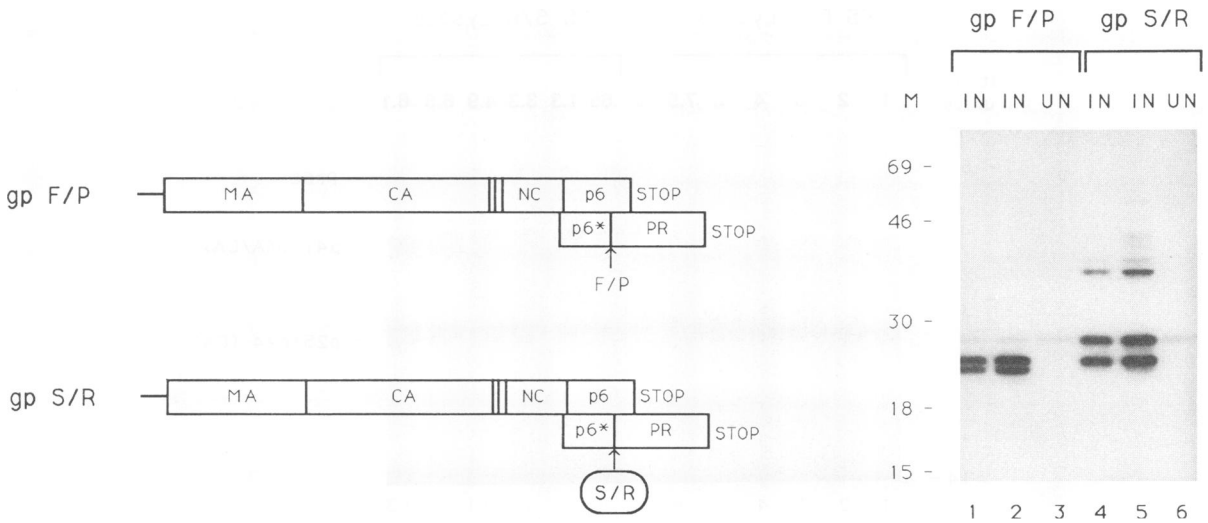

FIG. 7. (Left) Construction of vectors requiring ribosomal frameshifting for expression of wild-type PR (gp F/P) and N-terminally blocked PR (gp S/R). Open boxes represent proteins encoded by the HIV-1 sequences from nucleotide 333 to 2130 of BH10 (31). The nontranslated region (nucleotide 221 to 332) is indicated by a line. Proteins are as described for FS constructions (Fig. 1). In addition, the p6 domain at the C terminus of Gag is expressed in gp. (Right) expression of gp F/P and gp S/R in *E. coli* BL21(DE3). Bacteria were transformed with gp F/P or gp S/R and induced with IPTG. The proteins in total cell lysates were separated by SDS-PAGE (12.5% gels), immunodetected by Western blotting with polyclonal antibody against CA protein, and visualized by ECL. Lanes 1 and 2, induced lysates of bacteria transformed with gp F/P; lanes 4 and 5, induced lysates of bacteria transformed with gp S/R; lanes 3 and 6, uninduced lysates. Lanes 2 and 5 contain twice the amount of lysate loaded in lanes 1 and 4, respectively.

primarily of uncleaved $Pr55^{gag}$ with smaller amounts of MA-CA (p41) and another intermediate probably corresponding to a Gag polyprotein with the p6 domain removed. It should be noted that substitution of S-R for F-P in the *pol* frame also changes the amino acid sequence in the *gag* reading frame in the p6 domain. However, the change occurs in a region of the p6 domain that was found to be nonessential for mature particle assembly (7).

Since the observed impairment of processing of Gag substrates cannot be attributed to reduced enzymatic activity of the enzyme containing a proline-to-arginine mutation at position 1 (Table 2), the results suggest that mutations that block release of mature PR decrease the efficiency of processing of Gag substrates. Moreover, a mutant containing only a single-site amino acid substitution at the P1 position of the scissile bond (gp F/P to gp S/P) also showed impaired Gag processing in the cytoplasm (Fig. 8A, lane 3) and, to a lesser extent, in particles (Fig. 8B, lane 3). In contrast to S-R sites, S-P cleavage

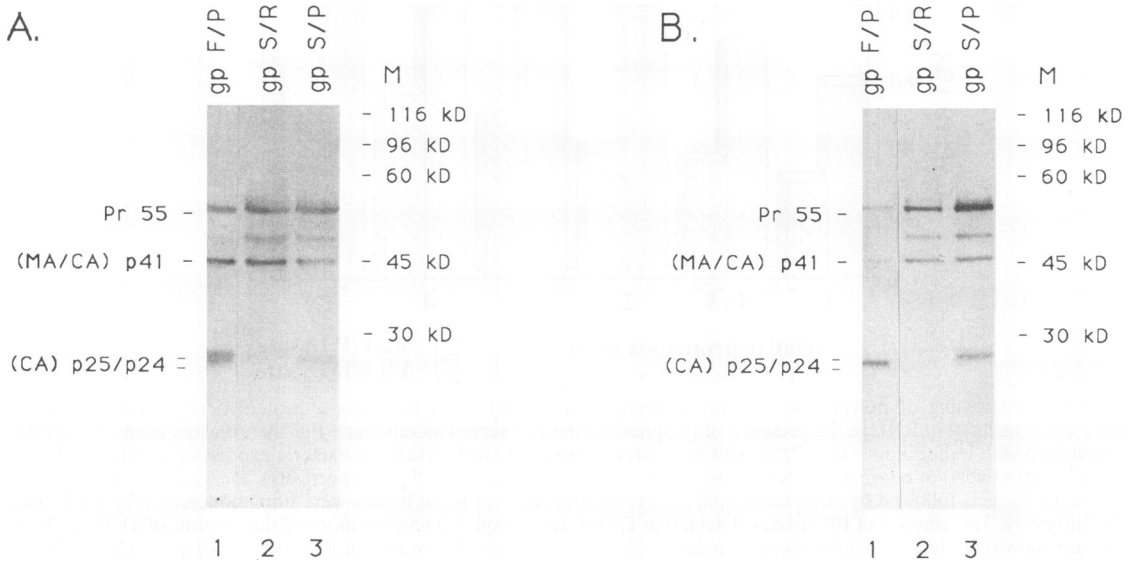

FIG. 8. Expression of gp in transfected mammalian cells. COS 7 cells electrotransfected in suspension were plated on 10-cm dishes and incubated for 48 h. Cells were harvested by scraping from the plate and lysed in SDS. Particles were obtained by sedimentation of cleared media through a cushion of sucrose. Proteins in cell (A) or particulate (B) extracts were separated by SDS-PAGE (17.5% gels) and analyzed by immunoblotting with polyclonal antiserum against CA protein.

sites can be hydrolyzed with a 50-fold molar excess of PR (26). It is likely that the inhibition of processing in the cytoplasm of cells transfected with gp S/P is overcome after concentration of assembly components in the particle. In any event, the results demonstrate that processing of Gag can be diminished even in the presence of the wild-type sequence at the P1′ position in PR and provide support for the conclusion that release of mature PR is necessary for efficient processing of Gag polyproteins.

DISCUSSION

Our results suggest that release of mature PR is not a prerequisite for processing of the HIV Gag-Pol precursor. This observation was also reported recently by Kotler et al. (16). In contrast, the Gag precursor form of Rous sarcoma virus PR is inactive (1). However, although our results show that Gag-PR autoprocessing is only slightly less efficient in vitro (Fig. 1) and in *E. coli* (Fig. 2), processing of the Gag precursor into mature products in *E. coli* (Fig. 7) and in mammalian cells (Fig. 8) was severely impaired when release of mature PR from Gag-Pol polyproteins was blocked. Although the mutated form of PR in *E. coli* and in COS 7 cells may not be identical, these results predict that in the absence of PR maturation, extensive premature processing of Gag by Gag-Pol precursors and cleavage intermediates would be unlikely.

Our studies also reveal several properties of the process leading to PR maturation. The fact that autoprocessing occurred in vitro and in *E. coli* strongly suggests that the enzyme was folded in a functional state, that PR activity did not require a free N terminus, and that mature PR was not required to initiate processing of cleavage sites in the Gag portion of the Gag-Pol precursor. These conclusions assume that the substitution of S-R for F-P at the N terminus of the PR domain in the precursor effectively blocked cleavage at this site. Several observations support this assumption. Incubation of the mutated polyproteins with a 50-fold excess of purified enzyme failed to generate an 11-kDa form of the enzyme (Fig. 3), and the two smallest products detected in induced bacterial cells had N termini located 8 to 10 residues upstream of the wild-type cleavage site or within the PR domain. In addition, Göttlinger et al. have shown that mutation of the MA-CA and CA-p2 junctions to S-R completely blocked cleavage at these sites (8). Substitution of arginine per se did not impair PR since (i) extended forms of PR containing this mutation were as effective as the 11-kDa mature PR in peptidolysis (Table 2) and (ii) mutation of the P1 position only (gp S/P) also resulted in a defective phenotype. All of the constructs used in this study lacked reverse transcriptase and integrase domains in Pol. A definitive evaluation of precursor activity will require examination of full-length Gag-Pol, since PR with mutations at both N and C termini is more impaired than PR with a single alteration (21, 41). The results described in this study do not exclude the possibility that cellular enzymes contributed to initial cleavage events.

Our results suggest that the pathway leading to PR maturation may include several extended forms (normally transient) from which PR may be autoprocessed. Once PR matures from such intermediates, efficient processing of Gag precursors can occur. That the 14- to 18-kDa extended PR forms that accumulate in bacterial lysates failed to convert to smaller forms unless incubated at a high concentration indicates that PR maturation from these fragments by an intramolecular pathway is not efficient. HIV expresses PR by translational frameshifting, forming a polyprotein with ≈60 amino acids between the NC and PR domains. The structure of this transframe region (p6*) may be a principal determinant of PR maturation. The p6* domain contains few hydrophobic residues and is predicted to be exposed and flexible (27). The crystal structure of PR (39) indicates that the p6*-PR junction would not be accessible for intramolecular processing in Gag-Pol precursors containing functional (i.e., dimerized) PR domains. If the NC (10) and PR domains are structured in Gag-Pol precursors awaiting assembly, the p6* insert may loop outside of the polyprotein molecular surface and reduce dimerization of the PR domain while it is part of the precursor. Once inside the particle, when Gag-Pol precursors are forced close together and PR dimerization is facilitated, p6* may be accessible to inter- or intramolecular cleavage by dimerized precursor PR. Cleavage in the p6* region may be favored if protein-protein interactions of the Gag domain make cleavage sites in Gag less accessible. The fact that we detected relatively efficient autoprocessing in RRL and in *E. coli* suggests that the Gag domains of the precursors made in vitro and in *E. coli* are not tightly aggregated, and thus Gag cleavage sites may be more accessible. In HIV, PR-specific processing has been detected in the transframe region in vitro (this study) and in virions (10a). However, this region does not appear to be readily hydrolyzed (Table 1) and may occupy the substrate binding cleft of the attacking enzyme precursor and delay the extent of overall processing. The relatively poor k_{cat} of the L-Q site in p6* supports this model. We suggested previously that p6* may function as an inhibitor of PR activation because deletion of this region facilitated autoprocessing of recombinant HIV-1 Gag-PR precursors in vitro (27). Once cleavage in this region occurs, the block limiting overall processing would be removed, allowing efficient PR maturation followed by the cascade of proteolytic events.

It will be of interest to examine the effects of p6* mutation on PR dimerization, maturation, and particle formation.

ACKNOWLEDGMENTS

We thank I. Jayatilaka, Glen Phillipsberg, Deonia Simmons, and Anke-Mareil Traenckner for technical assistance and C. Helmke for photography. L. Ehrlich and S. LeGrice provided helpful advice. We are grateful to L. Henderson, J. Kay, B. Dunn, and L. Phylip for many exciting discussions and for sharing their results prior to publication. We are particularly indebted to L. Phylip and J. Kay for performing the analyses described in Table 2.

This work was supported by NIH grant AI25993 (C.C.) and by a grant from the Bundesgesundheitsamt (FKZ III-007-09) (H.-G.K.). Peptide analyses were conducted by the Center for Analysis and Synthesis of Macromolecules at Stony Brook, which is supported by grants from NIH (RR02427) and the Center for Biotechnology.

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