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Mutational Analysis of a Native Substrate of the Human Immunodeficiency Virus Type 1 Proteinase

KATHRYN PARTIN,* HANS-GEORG KRÄUSSLICH,† LORNA EHRLICH, ECKARD WIMMER, AND CAROL CARTER

Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794

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Proteolytic processing of the *gag/pol* precursor by the human immunodeficiency virus type 1 proteinase is essential for the production of infectious viral particles. Although the sites of virus-specific cleavages have been determined, the primary amino acid sequences surrounding these sites are hetereogeneous and the determinants that direct the cleavage specificity exhibited by human immunodeficiency virus type 1 proteinase remain largely undefined. We performed mutational analysis of the Tyr/Pro site, which produces the amino terminus of the viral capsid protein, and the Phe/Pro site, which produces the amino terminus of the proteinase. Mutations were made in a clone encoding a frameshift mutation that results in the expression of equimolar amounts of the substrate and proteinase in the form of a truncated *gag/pol* precursor. After single-amino-acid substitutions were made, their effects on proteolytic processing were examined by in vitro transcription and in vitro translation of the synthetic mRNA; translation products were then processed by exogenously added purified proteinase. Single-amino-acid substitutions yielded both substrates which were processed with wild-type efficiency and substrates on which processing was impaired. At the Tyr/Pro site in *gag*, processing was severely inhibited by substitutions within the P4, P2, P1, and P2′ positions. The Phe/Pro site in *pol*, however, demonstrated far greater tolerance to amino acid substitution. These data suggest that the primary amino acid sequence around a scissile bond is more critical for cleavage of the Tyr/Pro site than the Phe/Pro site.

Proteolytic processing by human immunodeficiency virus type 1 (HIV-1) proteinase (PR) is an essential step in the maturation of infectious viral particles (for reviews, see references 7 and 17). Synthesis of large precursor polyproteins (either *gag* or a *gag/pol* fusion) and subsequent specific processing by the viral PR yield the mature structural components (matrix [MA], capsid [CA], and nucleocapsid [NC]), as well as the enzymes involved in viral replication (PR, reverse transcriptase [RT], and integrase [IN]) (20). Viral genomes which encode a defective PR produce virions with atypical structures which are noninfectious (12, 13, 33). It is likely, therefore, that specific inhibitors of PR may prove to be effective antiviral agents, which may be useful in the treatment of acquired immunodeficiency syndrome (5, 22).

The rational design of antiviral drugs directed against PR requires an understanding of the structure of PR and the mechanism by which it interacts with and catalyzes cleavage of the substrate. Primary sequence analysis of HIV-1 PR suggests that it belongs to the family of aspartic proteinases, although the polypeptide encoded by the retroviral genome represents only one-half of the enzyme (32). Experimental evidence supports this hypothesis, because HIV PR is inhibited by agents which are known to inhibit aspartic proteinases (11, 15, 35, 37). Furthermore, mutation of Asp-25 (the aspartic acid residue within the inferred active site, DTG) renders the protein inactive (13, 19, 21, 25, 37). Recently, the three-dimensional crystal structures of Rous sarcoma virus PR (24) and HIV PR (27, 43) were determined and confirm the structural similarity between the dimeric viral polypeptides and the cellular aspartic proteinases. There appears to be conformational similarity among aspartic proteinases (38, 39), which suggests that the catalytic apparatus in all aspartic proteinases is virtually the same. Therefore, it has been suggested that functional selection of the specificities of these enzymes evolved through changes in the substrate binding cleft (30), which directs the interaction between subsites of the enzyme and the side chains of the substrate.

HIV-1 PR cleaves at several unique sites which are overtly dissimilar, and no consensus sequence for retroviral PR recognition has been deduced (1, 8, 27, 28, 31, 40). Molecular modeling of HIV PR and its substrate has been used to predict subsites within the enzyme which would interact with the substrate, assuming that seven residues of a substrate may lie within the binding cleft (2, 42). Studies with purified PR and small peptides which mimic known cleavage sites have shown rates at which the different peptides are processed (14, 15, 41). Similar studies have led to the prediction that substrate determinants lie within seven amino acids flanking the scissile bond (3, 14). Mutational analysis by dipeptide substitutions of amino acids at scissile bonds in the *pol* gene demonstrated a distinct sequence requirement for cleavage (21). However, the exact determinants of substrate specificity appear to be complex and remain largely undefined.

The purpose of this study is to define further the determinants of substrate specificity of HIV PR. We have performed mutational analysis of the Tyr/Pro cleavage site, which produces the amino terminus of CA, and the Phe/Pro site, which produces the amino terminus of PR. Rather than using small peptides, we used an in vitro system which permitted us to evaluate the effect of a mutated site within the context

* Corresponding author.
† Present address: Institut für Virusforschung, Deutsches Krebsforschungszentrum, Im Neuenheimerfeld 280, D-69 Heidelberg, Federal Republic of Germany.

of its natural precursor. Results from this analysis of the *gag* Tyr/Pro site permitted us to determine the number of amino acids flanking the scissile bond which interact with PR and the relative importance of each amino acid position which lies within the binding cleft. The Phe/Pro site in *pol* appeared to be far more tolerant of amino acid substitutions.

MATERIALS AND METHODS

Plasmid construction. The construction of the parent plasmid (pHIV-FSII) has been described previously (16). pHIV-FSIII was constructed by transferring the *Eco*RI fragment of pHIV-FSII, containing nucleotides (nt) 221 through 2130 of the HIV cDNA (from BH10) (34) into pBS/KS (Stratagene) (29). Both pHIV-FSII and pHIV-FSIII contain a 4-base-pair (bp) insertion at nt 1640 of the HIV cDNA and two stop codons 3′ to the PR coding sequence (16). Plasmids were propagated in *Escherichia coli* BH 71-18.

In vitro transcription and translation. For in vitro transcription, the DNA was linearized at the *Eco*RV site in the pBS polylinker, 3′ to the coding sequences, and runoff transcription reactions containing 2.5 to 5 μg of linearized DNA and T7 polymerase were performed as previously described (16). Synthetic RNAs were translated in rabbit reticulocyte lysates (RRL; Promega), following the protocol of the supplier, in the presence of [^{35}S]methionine (specific activity, 1,100 Ci/mmol) (Dupont, NEN Research Products). Reactions were stopped by being chilled on ice, and samples were analyzed on a sodium dodecyl sulfate–10% polyacrylamide gel (SDS–10% PAG); the gel was then subjected to fluorography (En^{3}Hance; Dupont, NEN Research Products).

Site-directed mutagenesis. Mutagenesis was performed by the method of Kunkel (18) with minor modifications. Oligonucleotides of 17 to 18 bases in length containing a single base substitution were synthesized and hybridized to single-stranded pHIV-FSIII DNA that was enriched in uracil by being passaged through a *dut/ung* mutant host. Single-stranded DNA was purified from transformants as described by Kunkel (18) and subjected to sequence analysis by using the Sequenase Kit (U.S. Biochemical Corp.) and [^{35}S]dATP (Dupont, NEN Research Products; specific activity, 1,300 Ci/mmol). Products were analyzed by electrophoresis on an 8% Tris–borate–EDTA-urea gel at 80 to 100 W on an IBI apparatus. Gels were fixed, dried, and exposed to Kodak XAR film for 8 to 24 h.

Purification of PR and proteolytic processing in *trans*. Purification of PR was performed as described previously (15). In the *trans* reactions, 1 μl of purified PR was added to a translation lysate, in 50 mM morpholineethanesulfonic acid (MES, pH 5.9) and 1 mM EDTA, in a final volume of 30 μl. Reactions were incubated at 30°C for 1 to 16 h and analyzed by SDS-PAG electrophoresis (SDS-PAGE) as described above.

Peptide cleavage assay. Decapeptide cleavages were assayed by the methods of Kotler et al. (14). Ten-amino-acid peptides were synthesized (Multiple Peptides Systems), and their purity was checked by high-pressure liquid chromatography. Ten micrograms of peptide was incubated for 0 to 30 min in the presence of 10 μl of purified proteinase in a reaction volume of 50 μl. Samples were removed at different time points, and the reactions were stopped by being frozen in a dry ice bath. Two microliters of each sample was spotted onto a silica plate (EM Science) and was electrophoresed at 45 mA for 25 min. Plates were dried and sprayed with a 1% triethanolamine–acetone solution and were then sprayed with a solution of 0.1 mg of fluorescamine per ml of acetone. Plates were then exposed to UV light and photographed.

Immunoprecipitation analysis. Immunoprecipitation analyses were performed with antibodies against CA (10 μg/ml; Du Pont Co.), MA (20 μg/ml; Du Pont Co.), p6* (provided by Mary Graves, Hoffmann-La Roche), and PR (provided by Phillip Barr, Chiron). One to 5 μl of RRL was diluted 100-fold in buffer containing 1% SDS and 10 mM dithiothreitol, heated to 100°C for 2 min, and brought up to a 1-ml volume. Samples were preabsorbed with IgSorb (The Enzyme Center) for 15 min at 4°C and were then incubated with antibody for 1 h. If mouse antibodies were used, goat anti-mouse immunoglobulin G was added and the mixture was incubated for an additional 30 min. IgSorb was added and incubated for 30 min at 4°C. Antigen-antibody-IgSorb complexes were collected by centrifugation, washed, suspended in SDS sample buffer, and analyzed on an SDS-PAG as described above.

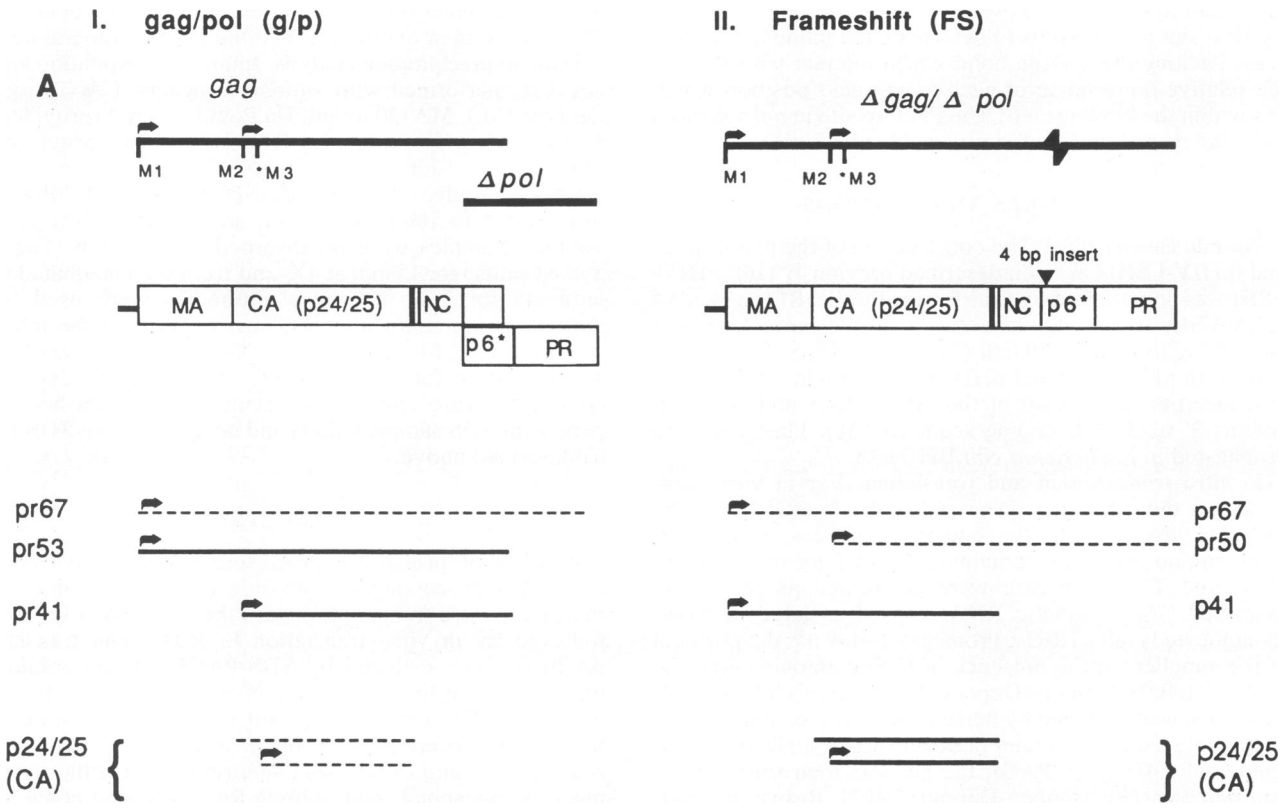
RESULTS

Analysis of proteolytic processing in vitro. Analysis of proteolytic processing was possible after expression of plasmids by in vitro transcription with the T7 RNA polymerase, followed by in vitro translation in RRL. The translation products were analyzed by SDS-PAGE and autoradiography. Our analysis was facilitated by the construction of a wild-type (WT) forced frameshift (FS) mutant, which contains a 4-bp insertion at nt 1640, resulting in a shift of the reading from *gag* to *pol* (16) and circumventing the requirement for ribosomal frameshifting for expression of PR (10). Figure 1A shows a schematic diagram of the HIV-1 sequences expressed in the FS construct and the *g/p* construct (containing the same sequences without the 4-bp insertion). Figure 1B shows an autoradiograph after translation of the two parental clones, WT *g/p* and WT FS. Both constructs contain two stop codons at the carboxyl terminus of PR (nt 2130). The effect of introducing the forced frameshift is readily seen by the efficient production of CA (p24/25) in WT FS as compared with that in WT *g/p* (compare lanes 1 and 2).

Two precursors are predicted to be synthesized from the WT *g/p* parental clone, the authentic *gag* precursor (pr53) and the precursor made after ribosomal frameshifting (pr67) (Fig. 1A, panel I; Fig. 1B, lane 1). Because pr67 is synthesized in very low amounts and is readily processed by PR, little pr67 accumulates upon the translation of WT *g/p*. In addition to the predicted precursors, a third precursor (pr41) was seen after translation of WT *g/p*. Translation of WT FS RNA (Fig. 1B, lane 2) produced the predicted *gag/pol* precursor, pr67, which was efficiently processed to a p41 intermediate and the p24/25 product. Because the proteins are labeled with [^{35}S]methionine, MA (which contains no Met) is not seen in this system. Under conditions which permitted less efficient processing of WT FS (see Fig. 6), an additional precursor, pr50, was also seen.

The additional *g/p* and FS precursors could be explained by utilization of Met-142 for internal initiation of translation, as has been reported in vivo (23). To determine the extent to which internal initiation occurred in our *g/p* and FS constructs, two mutations were constructed in which either the first Met (Met-1) or the second Met (Met-142) in *gag* was changed to Ile. The translation products of these mutants are shown in both the *g/p* construct, where limited processing by the endogenous PR occurred, and the FS construct, in which all substrate mutations were made.

Mutations of the first Met (Met-1→Ile) in both *g/p* and FS



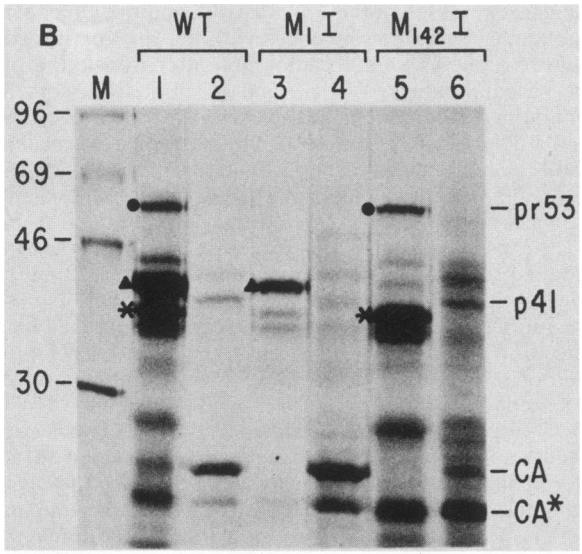


FIG. 1. Construction of native *gag* substrates either requiring ribosomal frameshifting (*g/p*) or permitting equimolar expression of PR (FS or forced frameshift). (Panel I) (*g/p*) The boldface bars represent the genes encoded by these sequences. The open boxes represent proteins encoded by the HIV-1 sequences that are included in the *g/p* clone (nt 220 through 2130 of BH10) (see Materials and Methods). These include 100 bp of the nontranslated region, the *gag* gene including MA, CA, NC, and p6, and, in the *pol* gene, p6* and PR. Bent arrows show the two major sites of initiation of translation. M1, Met-1; M2, Met-142; *M3, Met-171. The bottom of the panel shows the major gene products found after in vitro translation of synthetic mRNAs from this clone. Dashed lines represent products which were seen in lesser quantity. The pr67 species represents the *gag/pol* precursor polyprotein, seen in minor amounts because it was synthesized only after a frameshifting event. pr53 represents the *gag* precursor initiated at the first Met (Met-1). pr41 represents the truncated *gag* precursor initiated at the second Met (Met-142). The p24/25 band represents two CA species that were inefficiently produced in this clone; one in which the amino terminus is produced after cleavage at the MA-CA junction at amino acids 132 to 133, and the other in which the amino terminus is produced from initiation of translation at Met-142. (Panel II) (FS) The same sequences were used in the FS construct, except here a 4-bp insertion was made, shown as an inverted closed triangle. Because of the insertion, only the truncated *gag/pol* fusion precursor was translated, and from it, MA (p17), CA (p24/25), a slightly truncated NC, p6*, and PR were made. The pr67 precursor is represented as a dashed line, because it was seen in very low quantities as it was rapidly and efficiently processed to p41 and CA. pr50 is the *gag/pol* fusion precursor initiated from the second methionine. The processed p41 (MA-CA) is distinct from the pr41 (CA and NC) seen in the *g/p* clone, in that pr41 is due to an internal initiation at Met-142. (B) Analysis of internal initiation of translation. An autoradiograph of the translation products of WT and two substitution mutants is shown with the *g/p* and FS constructs. Lanes: 1, WT *g/p*; 2, WT FS; 3, Met-1→Ile *g/p*; 4, Met-1→Ile FS; 5, Met-142→Ile *g/p*; 6, Met-142→Ile FS. The following three precursors are labeled on the right side of the autoradiograph: pr53 (●) (originating from Met-1), prMet 2 (▲) (originating from Met-142), and pr*Met 3 (*) (presumably originating from Met-171). The p41 intermediate and CA product are also labeled. CA* refers to a capsid species which presumably originated from the utilization of Met-171 and therefore migrates substantially faster than CA derived from Met-1 and Met-142. Numbers on the left refer to molecular sizes in kilodaltons.

are shown in Fig. 1B (lanes 3 and 4, respectively). As predicted, the authentic *gag* precursor, pr53 is absent in *g/p* Met-1→Ile. The precursor initiated from Met-142 is seen, as well as are two other minor bands, presumably derived from

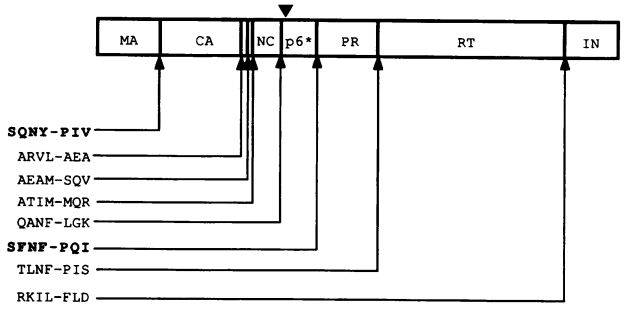


FIG. 2. Sequences in the HIV-1 *gag/pol* precursor that are cleaved by HIV-1 PR. Open boxes represent the proteins generated from the *gag/pol* fusion precursor. Arrows point to the sites where cleavage by HIV-1 PR is seen. The closed inverted triangle refers to the 4-bp insertion described in the legend to Fig. 1. Seven amino acids (shown in single-letter code) flanking each of these scissile bonds are listed by using the sequence from BH10. The boldface sequence (SQNY-PI) which is found at the junction of MA and CA, and SFNF-PQI, which is found at the junction of p6* and PR, are the sites analyzed in this study.

initiation at other internal initiation sites (from Met-171, etc.). In FS Met-1→Ile, efficient processing occurs and the precursors are not seen. Lanes 5 and 6 show the results of mutating the second Met, Met-142→Ile. The two predominant *g/p* precursors seen are initiated from Met-1 (pr53) and *Met3 (presumably Met-171, although this has not been confirmed by mutagenesis of Met-171). In FS Met-142→Ile, processing yields the p41 intermediate and CA. Most of the CA produced migrates more rapidly than does CA produced from WT FS because of the very efficient utilization of internal initiation from the third Met, which would yield a CA species that is missing the first 29 amino acids. The Met-142→Ile mutations demonstrate that efficient internal initiation occurs from whatever next Met is available; thus, a strategy of cloning substrate mutants into a construct devoid of internal initiation would not be possible. The efficiency of translation from Met-1 versus the next available Met appeared to be a function both of RNA concentration in the translation reaction and of the nature of the upstream noncoding leader sequences present on the mRNA (G. Zybarth and C. Carter, data not shown). It should be of interest to determine whether use of the precursor initiated from Met-142 and subsequent production of the amino terminally truncated CA contributes to the formation of viable virus particles in vivo.

p41, the intermediate seen in WT FS and FS Met-142 Ile, does not accumulate in FS Met-1 Ile, confirming that p41 is a processed intermediate which is derived only from initiation at Met-1. The 41-kilodalton intermediate contained a blocked N-terminus preventing amino acid determination (data not shown) and was recognized by monoclonal antibodies against MA and CA (see Fig. 6), consistent with it being p41, a previously reported processing intermediate (16, 23).

Effects of single-amino-acid substitution of the Tyr/Pro cleavage site in *gag*. HIV-1, like other retroviral proteinases, must process at several sites within *gag* and *pol*. Unlike cellular and some RNA virus proteinases (17), neither the amino acids constituting the scissile bond (the peptide bond which is cleaved) nor the flanking amino acids which interact in the binding cleft of PR are identical in primary sequence (Fig. 2). We asked whether one could identify some of the substrate determinants recognized by PR by using site-directed mutagenesis of the amino acids surrounding and including the Tyr/Pro scissile bond, which generates the amino terminus of CA. This site was chosen because CA is the major component of the virion structure, making its production of clear biological significance, and because PR uses a single, unambiguous cleavage site at the amino terminus of CA. All substrate mutations are shown in the FS (rather than *g/p*) constructs.

Because peptide data suggested that specificity determinants lay within seven amino acids flanking the scissile bond, we made two different single-amino-acid substitutions at every position from P6 to P5′ (36) with respect to the Tyr/Pro scissile bond. One mutation was predicted to be "functionally conservative" and one was to be "radical", based on Dayhoff's ranking of the susceptibility of an amino acid to be changed through evolution (4). By these criteria, groups which are functionally conserved are as follows: (i) Ala, Pro, Gly, Gln, Asn, Glu, Asp, Thr, Ser; (ii) Val, Ile, Met, Leu; (iii) Lys, Arg, His; (iv) Cys; and (v) Phe, Tyr, Trp. Note that chemical similarity is not necessarily as valuable predictively as is size.

To identify substitutions which rendered the Tyr/Pro site resistant to processing, each mutant was transcribed in vitro and translated in RRL. The products of translation were incubated with purified HIV PR in *trans* assays and were then analyzed on an SDS-PAG followed by autoradiography (Fig. 3). A 50- to 100-fold excess of purified PR was added, on the basis of the estimated amount of [^{35}S]methionine-labeled precursor synthesized in the translation reaction mixture. Under these conditions, the WT substrate was processed completely, producing CA as the major labeled product (Fig. 3, lane WT). The CA species seen in this figure represents CA produced from precursors initiated at Met-1, Met-142, and Met-171 and thus was seen as a heterogeneous mixture. Many mutants were cleaved as efficiently as was WT in *trans*. However, six mutations in four positions blocked cleavage at Tyr/Pro and resulted in the accumulation of p41. This intermediate represents the MA-CA species expected to accumulate in the absence of processing of the Tyr/Pro site, since it reacted with monoclonal antibodies directed against MA and CA but not p6* or PR (data not shown). The resistant mutants were in positions P1 (Y-132→S and Y-132→P), P2 (N-131→Q and N-131→K), P4 (S-129→R), and P2′ (I-134→K). Mutations which did not impair processing were seen at positions five or more residues from the scissile bond on the P side and four or more residues on the P′ side. Frequently, a product of ca. 28 kilodaltons was seen in both WT and mutated substrate *trans* assays. This product reacted with αp24 antibodies and was seen sporadically after the translation and processing of identical synthetic mRNAs. These properties suggest to us that this 28-kilodalton (kDa) product is a translational modification of CA rather than an aberrantly cleaved intermediate. Substitutions generating impaired substrates lay between positions P4 and P3′, which is consistent with the results of previous studies defining a "window" within which specificity determinants lie (2, 3, 42).

Further distinctions between substrate positions within the seven-amino-acid window were revealed by substitution by conservative versus radical amino acids. Functionally conservative, but not radical, amino acid substitutions were permitted at P4, P1, and P2′, but neither change was tolerated at the P2 position. These results are summarized in Fig. 4. Positions around the scissile bond exhibited various degrees of stringency for particular amino acids ranging from

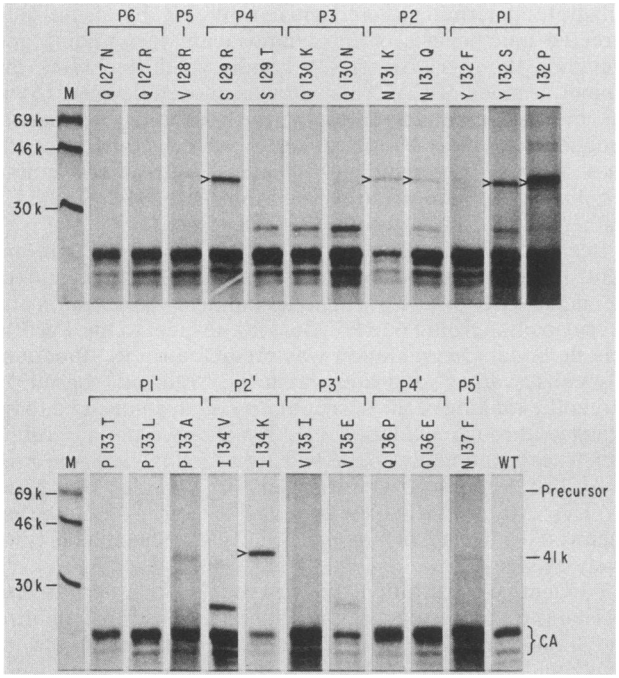


FIG. 3. Products of in vitro translation and PR processing after single-amino-acid substitution of the Tyr/Pro cleavage site in *gag*. Mutations were assayed after in vitro transcription and in vitro translation in RRL, followed by incubation with purified PR for 16 h at 30°C. Single-amino-acid substitutions were made by site-directed mutagenesis of the FS parent clone. Products were seen after the cleavage reactions were run on an SDS–10% PAG, followed by autoradiography. The position of the mutation relative to the Tyr/Pro scissile bond (marked by the boldface arrow) is indicated at the top of the figure. P*n* is the position from the amino side of the cleavage, and P*n*′ is the position from the carboxyl side. The nomenclature by which the mutants are described indicates the following: first, the WT amino acid residue; then, the position at which the residue resides in the protein sequence; and finally, the substituted residue. Thus, Q-127→N is a mutation of Asn for Gln at amino acid residue 127 in *gag*. The WT lane shows complete processing of all intermediates to CA species. A resistant 41-kDa band was detected in some mutations and is marked by a caret. Numbers to the left refer to molecular sizes in kilodaltons.

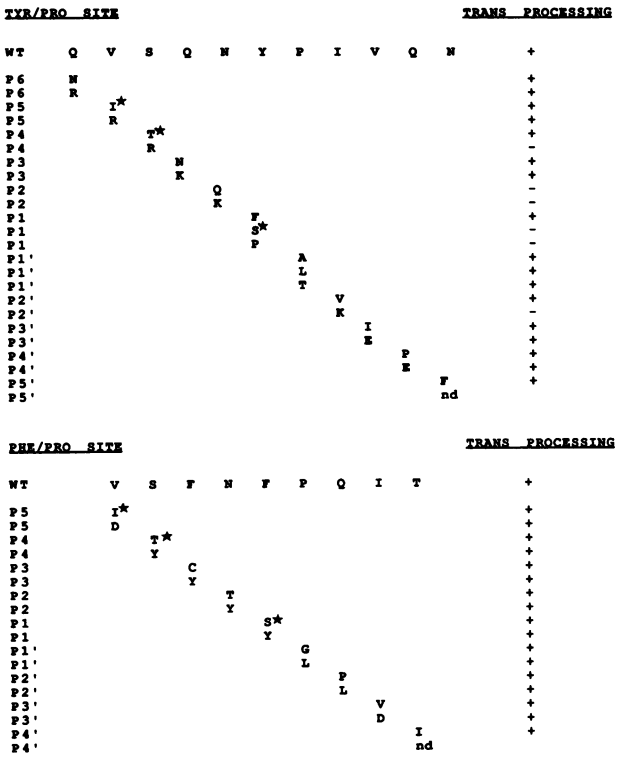


FIG. 4. Summary of mutations made around the Tyr/Pro and Phe/Pro cleavage sites. The positions around the Tyr/Pro and Phe/Pro scissile bonds at which mutations were made, as well as what these mutations are, and the phenotype after processing with purified PR are shown. Processing is measured by the absence of a resistant 41 species, as seen in Fig. 6 and 8, and is indicated by a plus sign; presence of the 41 species, which indicates impaired processing at the Tyr/Pro site, is indicated by a minus sign. Identical substitutions, which were made around both the Tyr/Pro site and the Phe/Pro site, are indicated by a star.

none (P6, P5, P4′, and P5′), low (P1′; see Fig. 6) or restricted (P4, P1, and P2′), to high (P2). The fact that two very different substitutions at P2 (N to K and N to Q) and P1 (Y to S and Y to P) had the same net effect suggests that other amino acid substitutions at those positions would result in similarly impaired processing.

Assaying decapeptides which encode a mutated Tyr/Pro site. To determine whether a single-amino-acid substitution which impaired processing of the MA-CA junction on a native substrate, producing p41, would block processing of a peptide substrate, decapeptides of the Tyr/Pro site were synthesized and analyzed. Three decapeptides were studied; one with WT sequence (VSQNY PIVQN), one with a mutation of P1 (VSQNS PIVQN), and one with a mutation of P2 (VSQQY PIVQN). The results from the P2 (N-131→Q) peptide cleavages are shown in Fig. 5. Either WT or N-131→Q peptides were incubated with purified PR, and samples were removed after various times. The samples were spotted onto a silica plate, and thin-layer electrophoresis was performed by a previously described technique (14). After electrophoresis, the silica plate was sprayed with fluorescamine and exposed to UV light. While the WT peptide was rapidly processed, the N-131→Q peptide was resistant to processing, even after 30 min (Fig. 5). The P1 mutation (Y-132→S) was also not cleaved by PR (data not shown) when assayed by thin-layer electrophoresis. We concluded from these experiments that mutations at the P1 and P2 positions did, in fact, result in uncleavable substrates, resulting in the accumulation of p41 when assayed on a native substrate and resulting in an uncleavable peptide when assayed by thin-layer electrophoresis.

Immunologic characterization of processing intermediates and products. RRL translations of FS constructs encoding the Tyr/Pro substrate mutations were also examined directly on an SDS-PAG, without processing by exogenous PR, to determine whether abnormal precursors or intermediates could be seen. Most mutations yielded the expected quality and quantity of the p41 intermediate and CA as compared with wild type (data not shown). One mutation, P-133→L, produced an altered level of p41 and CA relative to WT and accumulation of intermediates not seen with a WT substrate. However, P-133→L did not yield a Tyr/Pro site that was resistant when assayed with purified PR (Fig. 3).

Further analysis by immunologic characterization of WT and P-133→L products (Fig. 6) was done to define and

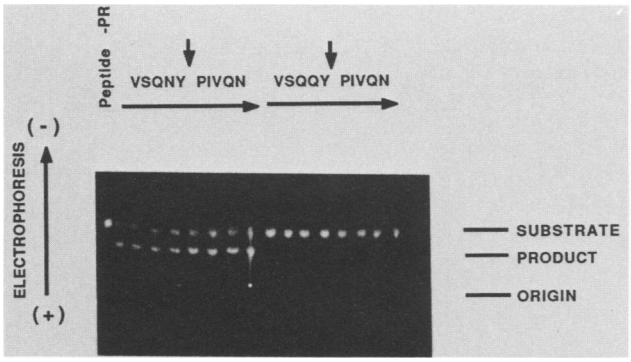


FIG. 5. Thin-layer electrophoresis of decapeptides processed with purified PR. Decapeptides of either WT (VSQNY PIVQN) or a mutation at the P2 position (VSQQY PIVQN) were incubated with purified PR for various times. Samples were removed and spotted onto a silica plate, upon which thin-layer electrophoresis was performed. Plates were treated with fluorescamine and were exposed to UV light. The direction of migration is indicated on the left. The first lane is a control, in which the peptide was incubated in the absence of PR. Each peptide was assayed after eight time points during incubation with PR for 0, 1, 2, 4, 8, 6, 32, and 60 min. To the right of the figure, the origin (where the peptides were spotted) and the final migrations of the intact substrate and its amino product are indicated.

compare the nature of the processing intermediates and products in an impaired substrate. The left side of Fig. 6 shows the analysis of WT products; the center panel schematically defines WT intermediates. In the WT FS translation (lane 0 in the WT panel), the RNA concentration was reduced relative to that seen in Fig. 1B, resulting in less efficient processing and therefore accumulation of larger intermediates and precursors. Immunoprecipitation of WT proteins with αp17 antibody (lane αp17 in the WT panel) allowed us to identify precursors initiated from Met-1, particularly pr67k (band a), the *gag/pol* fusion precursor; p56 (band b), the first intermediate resulting from PR excision at the carboxyl terminus of the polyprotein; and p41 (band d), the processing intermediate which contains p17 and p24 (MA-CA). The reduced signal from the αp17 antibody reflects its lower avidity in comparison with the other antibodies. Immunoprecipitation of WT proteins with αp24 antibodies (lane αp24 in the WT panel) showed that most intermediates and all precursors contained CA sequences. Immunoprecipitation with αPR antibodies (lane αPR in the WT panel) reacted with the intact precursor, pr67 (band a), and with three additional proteins. One of these proteins (band c), which did not react with αp17 but which reacted with αp24 and αPR, represented the internally initiated full-length precursor (pr50). The other two PR-containing intermediates are not readily identifiable as known intermediates.

The right panel of Fig. 6 characterizes P-133→L with the same antibodies. Direct analysis (lane 0 in the P-133→L panel) showed decreased p41 (band d) relative to increased amounts of novel intermediates (denoted by carets) not seen with WT. That several of these novel intermediates are immunoprecipitated with αPR is intriguing. It is unclear by what mechanism impairment of the Tyr/Pro site by P-133→L would inhibit the cleavage of the Phe/Pro site, which produces the amino terminus of PR.

Effect of single-amino-acid substitutions of the Phe/Pro site

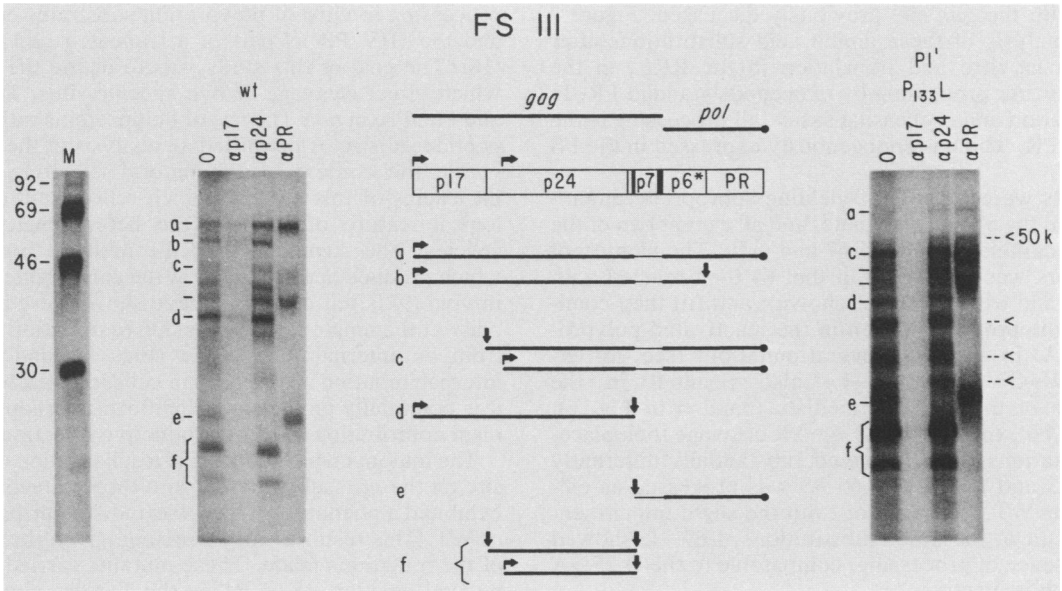


FIG. 6. Immunological characterization of processing products from a WT and a mutated cleavage site. Products were characterized further by their immunoreactivity to antibodies directed against HIV proteins. The WT gel in the left side of this figure shows no immunoprecipitation (lane 0), and immunoprecipitation against MA (lane αp17), against CA (lane αp24), and against PR (lane αPR). Products are identified by lowercase letters, which are also used in the middle panel to identify schematically what these bands most likely represent. The boldface lines in the center panel represent immunoreactivity against the protein in that region, and thin lines represent the region to which no antibodies were available for testing. In the center panel, bent arrows represent sites of initiation of translation, straight arrows represent cleavage sites, and the filled circles represent the termination of translation. The autoradiograph to the right shows the products of the mutant, P-133→L, after immunoprecipitation. The carets at the right of this gel mark the positions of the novel products seen in this mutant which are not detected in WT. Panel M shows molecular size markers in kilodaltons.

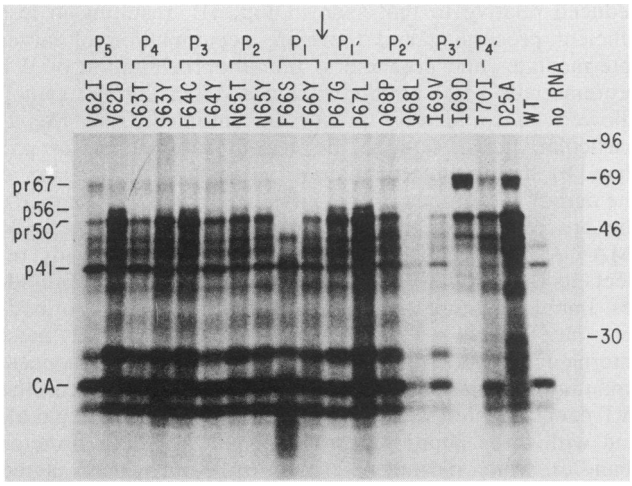


FIG. 7. Products of in vitro translation after single-amino-acid substitutions of the Phe/Pro site in *pol*. Single-amino-acid substitutions were made around and including the Phe/Pro site, which produces the amino terminus of PR. For a description of mutant nomenclature, see Fig. 3. After translation in RRL, products were analyzed on a 10% PAG followed by autoradiography. To the left of the autoradiograph are the following labeled species of interest: pr67, the intact precursor; p56, the intact polyprotein from which only PR has been cleaved; pr50, the intact precursor initiated from Met-142; p41, processing intermediate; and p24/25, the CA product. Numbers to the right indicate molecular sizes in kilodaltons.

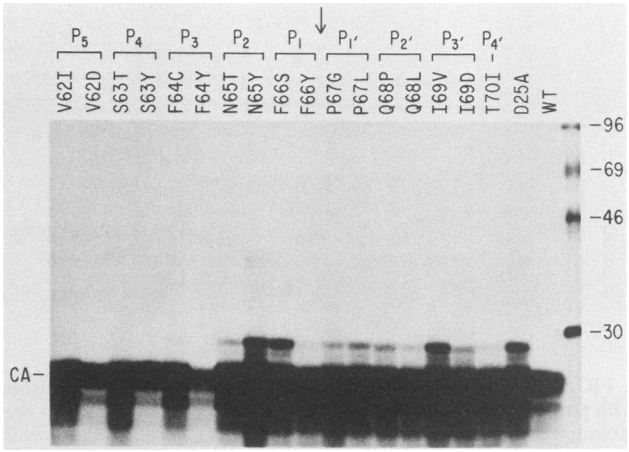


FIG. 8. Phe/Pro mutant products after being processed with purified HIV-1 PR. Autoradiography of the products of translation of the Phe/Pro site mutants is shown, after incubation with HIV-1 PR for 16 hours at 30°C. The only product seen is the CA species. Numbers to the right indicate molecular sizes in kilodaltons.

in *pol*. The amino terminus of PR itself is produced after cleavage of the Phe/Pro site (Fig. 2). Single-amino-acid substitutions were made around and including the Phe/Pro site to identify substrate determinants and to compare this site in *pol* with the *gag* site previously discussed. Figure 7 shows the analysis of these amino acid substitutions after transcription in vitro and translation in the RRL, in the absence of further processing by exogenously added PR. In this cotranslation and processing assay, all processing is the result of the PR, which is endogenously expressed in the FS constructs.

All mutants were capable of yielding appropriate quantities of CA and the p41 intermediate, and all except two of the mutations accumulated some pr67 and pr50. The identity of the precursors was confirmed in that (i) they reacted with αp17, αp24, and αPR (data not shown); and (ii) they comigrated with the precursors seen in the inactivated polyprotein, D-25→A. In addition, several mutations (see, for example, P-67→G and P-67→L) also resulted in the accumulation of the p56 intermediate (band b in Fig. 6), which would be expected if only the PR cleavage took place. The two mutations which behaved substantially differently were F-66→S and I-69→D. F-66→S was processed as efficiently as was WT, showing not even the slight impairment seen as a result of the other substitutions. I-69→D showed complete absence of processing, comparable to the D-25→A mutant, which is inactive.

To determine if any of the intermediates or precursors were completely blocked by mutations of the Phe/Pro site (as seen in Fig. 3 for the Tyr/Pro mutations), we processed the RRL products by adding excess purified PR in *trans*, as described previously (Fig. 8). Surprisingly, none of the Phe/Pro substitutions produced an uncleavable precursor or intermediate and all of the Phe/Pro substitutions were completely processed by the purified PR to their final CA products. As expected, the D-25→A mutation was capable of being processed to CA. The I-69→D mutation, which was completely inhibited in the cotranslation and processing reaction (Fig. 7) also was capable of being processed by the purified PR in *trans* (Fig. 8). None of the substitutions at positions which affected processing of the Tyr/Pro site affected cleavage at the Phe/Pro site.

DISCUSSION

We have previously demonstrated efficient and accurate processing in vitro of polyprotein substrates containing *gag* and the HIV PR as part of a truncated *gag/pol* precursor (16). The goal of this study was to define the determinants which direct cleavage of two specific sites, Tyr/Pro in *gag* and Phe/Pro in *pol*. The use of polyproteins rather than small peptide substrates permitted an analysis of the determinants within the context of the natural substrate. One of the intricacies of this system, which reflects an important biological feature of HIV, is the heterogeneity of the CA species. The significance of the multiple processing sites which produce heterogeneity at the carboxyl terminus of CA in vivo (9) is not clear. We have shown here that heterogeneity at the amino terminus is due to initiation of translation from an internal methionine (Fig. 1A and B). Because internal initiation is also seen in cells infected with HIV (23), it is potentially biologically significant, perhaps as a mechanism contributing to a nonproductive infection.

The mutants which contained a substitution of the Tyr/Pro site on the *gag* substrate fell into three categories. The first exhibited a phenotype which was indistinguishable from that of WT. This result is not surprising, given the subtle nature of the mutations made. These mutants carried substitutions beyond position P5 or P4′ in the Tyr/Pro site. The second category of mutated substrates exhibited impaired processing, such as P-133→L, a substrate which was defective but not inactive, and resulted in the accumulation of aberrant intermediates. Thus, the P-133→L substitution at P1′ yielded effects distal to the site at which it was made. Distal effects of substrate mutations have been described by Loeb et al. (21). These mutated sites could behave as competitive inhibitors, diminishing the effective concentration of PR and

thus subsequent processing. Alternatively, the substrate polyprotein could be folded in such a way as to permit ordered processing of the cleavage sites it encodes, and lack of processing at one site would prevent processing of the rest of the polyprotein. Conformational requirements of the polyprotein may play a role in the activation of PR, since, in vitro, activation is temporally and spatially regulated. It is interesting that all mammalian aspartic proteinases are encoded as zymogens, which are activated only after cleavage at a unique site from their proenzyme forms (39).

The third category of mutated substrates was completely defective for cleavage at Tyr/Pro in the presence of 50- to 100-fold excess PR and exhibited a resistant p41 species which represented the MA-CA domains. Thus, radical substitutions at P4, P2, P1, and P2′ positions, and even a conservative substitution at P2, produced an uncleavable Tyr/Pro substrate. When these mutations were incorporated into decapeptide substrates encoding the amino acid sequences of the Tyr/Pro scissile bond, they were not cleaved by purified PR in an in vitro cleavage assay.

The results of mutagenesis of the Phe/Pro site in *pol* did not follow the pattern of the Tyr/Pro mutations. Of the two mutations which caused an effect in the cotranslation and processing assay, one resulted in inactive enzyme (I-69→D) and one resulted in very efficient processing (F-66→S). That I-69→D was not a substrate defect was apparent from the *trans* assay, which showed that this site was completely cleavable by excess PR. It is unclear by what mechanism this mutation inactivated the enzyme. It is interesting to note that interactions between the P3′ substrate site and the S3′ subsite of PR are predicted to include Asp-25 from PR (42). Whether I-69→D interferes with this subsite and affects catalysis or whether it interferes with the catalytic Asp-Asp interaction remains to be tested.

The F-66→S mutation, which resulted in remarkably efficient processing, even in *trans*, was surprising in light of the results from a similar mutation at the Tyr/Pro site (Y-132→S). In both cases, the resulting dipeptide is Ser/Pro; however, in the context of the Tyr/Pro site, the Ser/Pro dipeptide blocks cleavage, while in the Phe/Pro context in a native substrate, it is quite a good substrate. We conclude from these two mutations that (i) the context of the substrate around the scissile bond is critical and (ii) the stringency for substrate determinants is much greater at the Tyr/Pro site than at the Phe/Pro site. Our conclusion is supported by the result that none of the Phe/Pro mutations rendered a resistant substrate, while six of the Tyr/Pro mutations did just that (Fig. 4). It may be further supported by a comparison of evolutionary sequence variation around the two sites (Fig. 9). Although the sequences around both the Tyr/Pro and the Phe/Pro sites are conserved, the Tyr/Pro site appears to be more constrained than does the Phe/Pro site. It may be, in fact, that conformation as well as primary sequence structure are determinants of specificity (40). Thus, the Tyr/Pro site may be restricted by sequence and conformation while the Phe/Pro site is primarily restricted by conformation. Such differences may not be discernable with peptide studies in which small (seven-amino-acid) sequences are studied.

The difference in the degree of stringency at the CA versus the PR site suggests that PR processing of the polyprotein is a regulatory event. One interpretation of these data is that inappropriately facile processing of the CA site would produce immature and thus noninfectious particles. This would be avoided by a strict requirement for substrate-subsite interactions. A second interpretation of these data is that processing of the amino terminus of PR is loosely restricted.

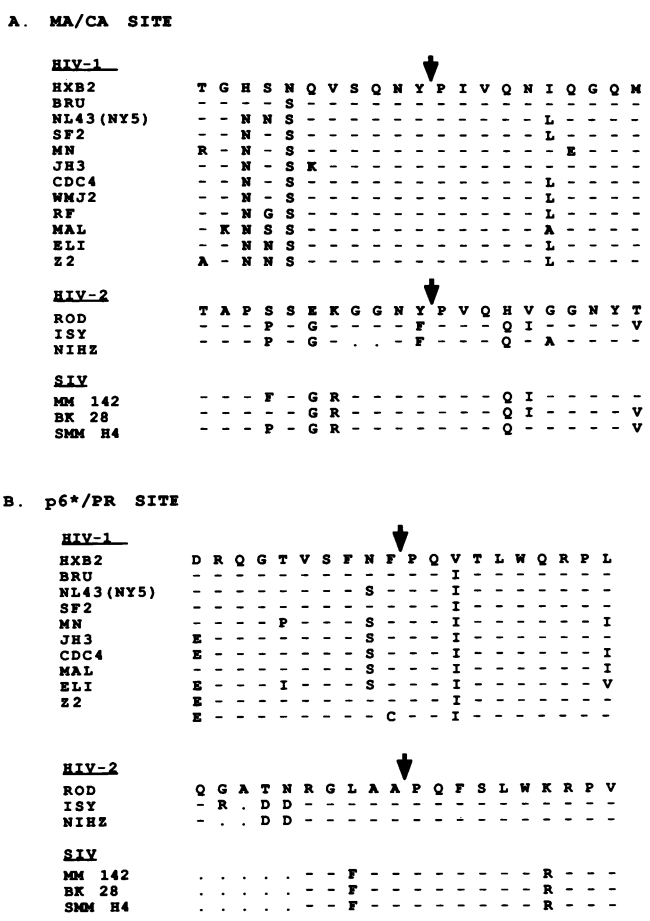


FIG. 9. Alignment of amino acids flanking the Tyr/Pro and Phe/Pro cleavage sites. Amino acid sequence alignments were taken from Myers et al. (26). Identical residues are indicated by dashes. Dots indicate gaps inserted to improve the alignment. HIV-1 alignments use HXB2 as the reference sequence. ROD is the reference sequence for HIV type 2 and simian immunodeficiency virus.

This could be necessary to allow autocatalysis, in that the bulky polyprotein from which PR is excised necessitates less stringent substrate-subsite interactions. These two (nonexclusive) interpretations could be tested by a mutagenesis study of the Leu/Ala or Met/Met sites which, when cleaved, form the carboxyl terminus of CA. One would predict a fairly high degree of stringency for substrate determinants at this site, and, in fact, preliminary data supports this (6).

The results of our studies may be applied to test definitions of a consensus PR cleavage site. On the basis of amino acid similarities around the peptide bonds cleaved by viral PRs, Henderson et al. (8) have suggested three classes of cleavage sites in the *gag/pol* polyprotein. Class I sites include the Tyr/Pro and Phe/Pro sites in HIV-1. They define the specific traits of these sites by a requirement for the following: (i) a hydrophobic residue at P2′, (ii) asparagine at P2, and (iii) glycine or polar amino acids at P3. Our results of mutagenesis of the Tyr/Pro site at these positions agree very well with the specifications which classify Tyr/Pro as a class I site. Moreover, our results suggest a high degree of stringency for the determinants at these positions. Our results from the Phe/Pro site suggest that this site does not fit functionally with class I sites, as predicted by the sequence analysis of Henderson et al. (8).

Loeb et al. (21) have mutagenized the P1 and P1′ positions of cleavage sites within the *pol* gene. They describe three classes of mutations which permit the following: (i) WT processing; (ii) absence of processing at the mutated site, but efficient processing at distal sites; and (iii) no processing at the mutated site or at distal sites. The first and second classes of mutants are similar in phenotype to those described in this report. No mutants phenotypically similar to those in class 3 were detected in our studies, except I-69→D, which was found by the *trans* assay to be a mutation which impaired PR activity rather than recognition of the substrate.

Our studies contribute to an understanding of determinants critical for PR processing. They suggest that the stringency of the requirement for these determinants is greater at the Tyr/Pro site in *gag* than at the Phe/Pro site in *pol*. Within the Tyr/Pro site, there appears to be an ordered hierarchy of amino acid positions which are critical for processing, including the P4, P2, P1, and P2′ positions. That the determinants of the Phe/Pro site are less stringent may be of biological significance and merits further study.

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