Point mutation in avian sarcoma leukaemia virus protease which increases its activity but impairs infectious virus production

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The retrovirus protease (PR), an aspartic PR, is composed of two identical subunits, each containing a conserved tripeptide sequence present at the active site of the enzyme. Asp-Ser-Gly is found in avian sarcoma leukaemia viruses (ASLV) and Asp-Thr-Gly in mammalian oncoretroviruses. We have mutated the conserved sequence at the active site of ASLV PR by converting the Ser and Gly residues to Thr and Ala, respectively. Replacement of Gly with Ala yielded an ASLV PR devoid of proteolytic activity. The Ser to Thr conversion did not alter the substrate specificity of the enzyme. Both wild-type and mutated PRs correctly cleaved viral precursors expressed in bacterial cells, as well as synthetic peptides homologous to ASLV and human immunodeficiency virus type 1 cleavage sites. Bacterially produced ASLV PR with Thr instead of Ser had increased enzymatic activity, as shown by hydrolysis of synthetic peptides. However, this mutation reduced the production of reverse transcriptase-containing particles and infectious virus following transfection of permissive cells with virus DNA.

Introduction

The gag and pol genes of retroviruses code for polyproteins that are subsequently processed by virus protease (PR). Both Gag and Gag–Pol polyproteins are translated from mRNA that is indistinguishable from the full-length genomic RNA found in virions. The ratio of Gag to Gag–Pol polyprotein is controlled either by frameshift or nonsense mutation suppression, depending on the particular strain of retrovirus or retrotransposon (Coffin, 1990).

The virus polyproteins are processed by a virus-encoded PR, which is a dimer composed of two identical subunits. Virus PR can be synthesized either as part of the Gag, Gag–PR or Gag–Pol polyproteins, depending on the retrovirus strain (Debouck et al., 1987; Kohl et al., 1988; Krausslich & Wimmer, 1988; Oroszlan & Luftig, 1990; Tomasseli et al., 1991; Wlodawer et al., 1989). In most mammalian retroviruses the PR is synthesized only as part of the Gag–Pol polyprotein and as, such, a minor component of the virion (for reviews see Krausslich & Wimmer, 1988; Oroszlan & Luftig, 1990; Tomasseli et al., 1991). In contrast, the PR of the avian sarcoma leukaemia virus (ASLV) is synthesized as part of both Gag and Gag–Pol polyproteins. Consequently, the ratio of PR to structural proteins in ASLV is approximately 20 times higher than in mammalian retroviruses (Tomasseli et al., 1991).

A comparison of the conserved amino acid triplet at the active sites of ASLV PR and its mammalian counterpart shows that the former contains Asp-Ser-Gly, while the latter has Asp-Thr-Gly at the corresponding position (Pearl & Taylor, 1987; Tomasseli et al., 1991; Wlodawer et al., 1989). The Hepadnaviridae, distant relatives of retroviruses, also synthesize viral polyproteins that are thought to be processed by a virus PR. The PR for hepadnaviruses has not been explicitly defined, but Miller (1987) described a putative PR with the amino acid triplet Asp-Ser-Ala at the active site.

ASLV PR mutants were constructed to determine whether replacement of Ser (position 38) with Thr at the active site would alter enzymatic activity and, if so, whether this change would interfere with virus maturation. We show that conversion of Ser to Thr in the ASLV PR indeed leads to an enzyme that is approximately 10 times more active than the wild-type (wt). This mutated enzyme has the same substrate specificity as the wt enzyme, as judged by its ability to cleave synthetic peptide substrates identical to the PR cleavage sites of ASLV and to human immunodeficiency virus type 1.
homologous segments from the mutants. To ensure that the mutations replacing a TCT codon with ACT and a GGA codon with GCA, respectively. A plasmid expressing inactive PR was constructed by Kotler

letters show those nucleotides that differ from the sequences in wt

Ala were: 2203-TATCACCGCGCTGcTcGACaCTGGAGCGGA-

contains an infectious ASLV DNA that carries the

promoter.

site-directed mutagenesis (Morinaga

sequences in the genome of RSV Prague-C (Schwartz

bacteriophage lambda, the ribosomal binding site and the translational

initiation site. The plasmid pSJHgag was derived from pT7-13.

Plasmid pSJHgag contained a

gag

expression of ASLV Gag proteins cloned in the pSJHgag plasmid (a
generous gift of J. M. Coffin, Tufts University, Boston, Mass., USA)

and its derivatives.

E. coli strain DR100 was used as the host for vectors containing both

wt and mutated ASLV DNAs.

Plasmids. The bacterial clone pEV-vr2 containing the ASLV PR was used to express wt and mutant PR and nucleocapsid–PR (NC–PR; Kotler et al., 1988a, b). These plasmids contained the P16.6 region from bacteriophage lambda, the ribosomal binding site and the translational initiation site. The plasmid pSJHgag was derived from pT7-13. Plasmid pSJHgag contained a Sacl–HindIII fragment (nucleotides 255–2740; Schwartz et al., 1983) from the Rous sarcoma virus (RSV) Prague-C genome that encodes the entire Gag polypeptide. Expression of ASLV gag in pSJHgag is under the control of the T7 polymerase promoter.

RCASneo vector. Plasmid pRCASneo (Hughes & Kosik, 1984) contains an infectious ASLV DNA that carries the neo gene in place of src, thereby introducing a selectable marker.

Mutagenesis of the PR active site. Mutations were introduced into the active site of ASLV PR (Pearl & Taylor, 1987; Toh et al., 1985) by site-directed mutagenesis (Morinaga et al., 1984). The synthetic oligonucleotides used for mutagenesis were homologous to nucleotide sequences in the genome of RSV Prague-C (Schwartz et al., 1983). The oligonucleotides used to replace the Ser residue with Thr and Gly with Ala were: 2203-TATCACCGCGCTGcTcGACaCTGGAGCGGA-

CA-2234 and 2205-TCACCCGCGCTGCAGCTGAGCGCA-

ATCC-2236, respectively. Underlined sequences indicate a TaqI cleavage site which was used to detect the presence of the mutation; lower case letters show those nucleotides that differ from the sequences in wt ASLV. The plasmids that contained PR

and PR

were made by replacing a TCT codon with ACT and a GGA codon with GCA, respectively. A plasmid expressing inactive PR was constructed by

replacing Asp with Ile at position 37 (a generous gift from V. M. Vogt, Cornell University, Ithaca, N.Y., USA; as previously described by Kotler et al., 1988a).

The HpaI–EcoRI fragment (2197 to 2320) of pSJHgag (Schwartz et al., 1983), which includes the active site of PR, was replaced by homologous segments from the mutants. To ensure that the mutations were indeed introduced into pSJHgag, plasmids were cut with either TaqI or EcoRV (for PR

The SacI–HpaI fragment (2496 bp) of pRCASneo, which includes gag, was replaced by the homologous fragments from the wt and

mutated pSJHgag vectors. Restriction enzyme analysis was used to identify the mutations in the newly constructed RCASneo vector.

Transfection of cultured QT-6 cells. Ten μg of each DNA were transacted into cells by the calcium phosphate technique (Demetrios & Welkie, 1984). The presence of viruses in the culture media was detected by a reverse transcriptase (RT) assay (Goff et al., 1981).

Analysis of virus particles released from transfected cultures. Culture media harvested 48 h post-transfection were cleared from cells and cell debris by centrifugation for 10 min at 10000 g. The virus particles were pelleted from the supernatant by centrifugation at 100000 g for 50 min using a R-65 Beckman rotor. Pellets were suspended in 100 μl of 10 mM-Tris–HCl pH 8.0 and 1 mM-EDTA; virus proteins were detected by immunoblotting as described previously (Kotler et al., 1988a).

Analysis of PR expressed in E. coli. E. coli (strains MC1061 and BU8049) carrying expression vectors was grown at 30 °C. Viral protein synthesis was induced by shifting the cultures to 42 °C for 4 h. Virus proteins were detected by immunoblotting as described elsewhere (Kotler et al., 1988a). Virus proteins expressed in E. coli were detected with rabbit sera directed against PR, NC and capsid (CA) proteins. Anti-PR and anti-NC were prepared by immunizing rabbits with the purified virus proteins expressed in bacterial cells (Harlow & Lane, 1988); anti-CA was prepared by immunizing rabbits with partially purified CA extracted from purified virus preparations.

Preparation of bacterial extracts. Induced E. coli cultures (1.75 ml) expressing wt PR (PR

and PR

were pelleted and suspended in 400 μl of 1 % (v/v) tolene, 10 mM-Tris–HCl pH 8.0, 5 mM-2-mercaptoethanol, 0.1 mM-EDTA and 0.1 % Triton X-100. Bacterial cell suspensions were vigorously agitated by vortexing (3 min) and incubated for 20 min at 37 °C. Unbroken cells and cell debris were then pelleted by centrifugation. Supernatants were freeze-dried and dissolved in 50 μl of water, of which 10 μl were used for the reactions.

Purification of PR. Bacterial cells expressing PR were collected by centrifugation and the pellets were suspended in 50 mM-dextrose, 10 mM-EDTA, 25 mM-Tris–HCl pH 8.0 and 4 mg/ml lysozyme. The suspensions were incubated for 10 min on ice and subjected to three 15 s bursts of sonication. The bacterial lysates were then centrifuged for 30 min at 10000 g. The pellets were dissolved in 6 M-guanidine hydrochloride pH 8.0, shaken for 1 h at room temperature and centrifuged for 10 min at 6000 g. The supernatants were acidified to pH 2 by the addition of trifluoroacetic acid (TFA). The dissolved proteins were fractionated by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a 19 × 150 mm μ-Bondapack C8 column (Waters Associates) with a linear gradient of 0–60 % acetonitrile in the presence of 0.05 % TFA in water as previously described (Copeland & Oroszlán, 1988). Fractions from the RP-HPLC column were lyophilized, dissolved in 2 M-guanidine hydrochloride and 100 mM-Tris–HCl pH 8.0 at a concentration of 200–300 μg/ml, and refolded as previously described (Tozser et al., 1992). Briefly, one volume of protein solution was diluted with two volumes of refolding buffer (20 mM-PIPES pH 7.0, 100 mM-NaCl, 10 % glycerol, 1 mM-EDTA). The fractions were dialysed against the refolding buffer for 4 h at 4 °C. Protein concentration was measured using kits purchased from Bio-Rad.

Peptide synthesis, purification and analytical procedure. Oligopeptides were synthesized and characterized as described previously (Copeland & Oroszlán, 1988; Kotler et al., 1988a, b, 1989). The PR assays were carried out in 20 μl 0.25 M-potassium phosphate buffer pH 5.6, 7.5 % glyceral, 5 mM-dithiothreitol, 1 mM-EDTA, 0.2 % NP40, 0.5 mM-NaCl, 20 pmol PR and 40 nmol synthetic peptide at 37 °C. (Kotler et al., 1989; Tozser et al., 1991). The reaction mixtures were incubated at 37 °C for 1 h, unless otherwise indicated. The reactions were stopped by addition of guanidine hydrochloride (6 M final concentration).

The reactions with bacterial extracts were carried out similarly, but
**Results**

Expression of mutated PR in *E. coli*

*E. coli* containing plasmids expressing ASLV PR<sup>38Ser</sup>, PR<sup>38Thr</sup> and PR<sup>39Ala</sup> were grown to mid-log phase at 30 °C. Expression was induced by shifting the cultures to 42 °C and cells were harvested 4 h later. Virus proteins were analysed by PAGE, transferred to nitrocellulose filters and detected with antisera to PR. Proteins which comigrated with the virus PR and reacted specifically with anti-PR sera were produced by the induced, but not by the uninduced cells (Fig. 1a). Both wt and mutated PR were produced in bacterial cells in comparable quantities, as determined by Western blot analysis of equal amounts of cell extracts using alkaline phosphatase-conjugated goat anti-rabbit serum (Sigma). PR represented 5–10% of total protein in the induced cells (Fig. 1b), based on staining with Coomassie brilliant blue.

**PR<sup>38Thr</sup> can cleave virus polypeptides correctly**

Fused NC–PR protein undergoes self-cleavage when it is expressed in *E. coli* cells. It was previously shown that cleavage does not take place if the active site of PR is disrupted by substitution of Asp with Ile at position 37 (PR<sup>37Ile</sup>; Kotler et al., 1988a). *E. coli* cell extracts of bacteria harbouring pNC-PR<sup>38Ser</sup>, pNC-PR<sup>38Thr</sup> or pNC-PR<sup>39Ala</sup> plasmids contained proteins with a molecular mass of around 25 kDa as predicted for the NC–PR fusion protein. The proteins reacted with anti-PR (Fig. 2a) and with anti-NC (Fig. 2b) sera. Proteins that comigrated with ASLV PR and ASLV NC could be detected with anti-PR or anti-NC sera in cells that expressed NC-PR<sup>38Ser</sup> and NC-PR<sup>38Thr</sup>, but not in cells expressing NC-PR<sup>39Ala</sup> or NC-PR<sup>37Ile</sup> (Fig. 2a, b).

DNA fragments coding for the mutated ASLV PR were also inserted into vectors that expressed the entire Gag polyprotein. Fig. 3(a) shows that a protein which comigrated with virus CA and reacted with CA-specific sera was produced in bacterial cells that contained the plasmid pSJHgag, which expresses wt ASLV Gag protein. CA protein was also released from Gag polyprotein that contained PR<sup>38Thr</sup>, but not from Gag polyproteins that contain PR<sup>39Ala</sup> or PR<sup>37Ile</sup> (Fig. 3a). As expected, expression of Gag containing PR<sup>38Ser</sup> or PR<sup>38Thr</sup> resulted in the release of free NC, but NC was not released from Gag proteins that contained either PR<sup>39Ala</sup> or PR<sup>37Ile</sup> (Fig. 3b).

**PR<sup>38Thr</sup> has a substrate specificity similar to wt PR**

To determine whether substitution of Thr for Ser at the active site of PR causes an alteration in the enzyme...
specificity, we prepared oligopeptides homologous to some of the cleavage sites of the ASLV Gag-Pol polyprotein. Fig. 4 shows that peptides homologous to X1/p10, p10/CA, X2/NC, NC/PR, PR/X3 and RT/IN (SP2, 3, 5, 6, 7 and 9, respectively) that were cleaved by PR$^{38 \text{Ser}}$ were also cleaved by PR$^{38 \text{Thr}}$, but not by PR$^{39 \text{Ala}}$ (data not shown).

We compared the specificity of wt PR to that of PR$^{38 \text{Thr}}$ on synthetic peptides, based on the cleavage sites in HIV-1 (Fig. 4). MA/CA, X1/NC, PR/RT and RT/IN peptides (SP1, 3, 6 and 7 respectively) were cleaved by PR$^{38 \text{Ser}}$ and PR$^{38 \text{Thr}}$. Amino acid composition and sequence determination of the hydrolysis products isolated by HPLC showed that both PRs cleaved the oligopeptides at the same site (Fig. 4c). In addition, the data presented in Fig. 4(b) indicate that PR$^{38 \text{Thr}}$ cleaves ASLV and HIV-1 oligopeptides more efficiently than the PR$^{38 \text{Ser}}$ enzyme.

**Comparison of proteolytic activities of PR$^{38 \text{Ser}}$ with mutated PRs**

PR$^{38 \text{Ser}}$, PR$^{38 \text{Thr}}$ and PR$^{39 \text{Ala}}$ were purified to homogeneity (see Methods). X2/NC synthetic decapeptide (SP5) of ASLV was used as substrate to compare the activity of purified PR$^{38 \text{Ser}}$ with the activities of purified PR$^{38 \text{Thr}}$ and PR$^{39 \text{Ala}}$ directly. The kinetics of hydrolysis of the X2/NC decapeptide (Fig. 5a) showed that PR$^{38 \text{Thr}}$ was approximately 10 times more active than PR$^{38 \text{Ser}}$, with an estimated turnover number of about 30 nmol/min. PR$^{39 \text{Ala}}$ was inactive and did not cleave any of the peptide substrates listed in Fig. 4(c).

The increased activity of PR$^{38 \text{Thr}}$ over PR$^{38 \text{Ser}}$, as shown in Fig. 5(a), may have resulted from differences caused by the refolding efficiency of the enzymes after purification. The experiment was therefore repeated using the soluble fractions of bacterial cells containing equal amounts of PR, as determined by Western blots. Fig. 5(b) shows that PR$^{38 \text{Thr}}$ was approximately eight times more active than PR$^{38 \text{Ser}}$, confirming the results presented in Fig. 5(a).

Using different concentrations of X2/NC peptide (SP5) as a substrate, we determined that the apparent $K_m$ values of PR$^{38 \text{Ser}}$ and PR$^{38 \text{Thr}}$ were 12 $\mu$m and 16 $\mu$m respectively (data not shown). These results indicate that replacing the Ser residue with Thr increased the specific activity of the enzyme, while replacing Gly with Ala completely inactivated the enzyme.
Mutated PR reduces the production of RT-containing particles released from transfected cells

The results described above demonstrated that virus precursors expressed in bacterial cells are correctly processed by PR\(^{38\text{Thr}}\) and PR\(^{38\text{Ser}}\), but not by PR\(^{38\text{Ala}}\). We therefore tested the effect of active site mutations on virus maturation. Parallel QT-6 cell cultures were transfected with 10 μg of plasmid DNA containing virus genomes coding for PR\(^{38\text{Ser}}\), PR\(^{37\text{Le}}\), PR\(^{38\text{Thr}}\) or PR\(^{38\text{Ala}}\). Twenty-four and 48 h post-transfection culture media were harvested and assayed for the presence of RT activity. Fig. 6 shows that RT-containing particles were released from cells transfected with PR\(^{38\text{Ser}}\) virus DNA, but not with virus DNAs containing mutated PR\(^{37\text{Le}}\) and PR\(^{38\text{Ala}}\). Cells transfected with virus DNA expressing PR\(^{38\text{Thr}}\) released significantly lower amounts of RT-active particles.

Culture media were harvested from transfected cells (48 h post-transfection), concentrated by centrifugation and virus proteins in the pellets were detected by immunoblot analysis using anti-CA serum (Fig. 7). Particles containing unprocessed Gag (p76) were released from cells transfected with mutated viral PR\(^{38\text{Ala}}\) DNA. Particles with PR\(^{38\text{Ser}}\) contained only mature CA (p30), indicating that virus Gag precursors were completely processed. However, particles released from cells transfected with viral PR\(^{38\text{Thr}}\) DNA contained both unprocessed Gag precursor and mature CA proteins. These results may explain why particles containing PR\(^{38\text{Thr}}\) have reduced RT activity.

Viruses harvested 24 h post-transfection were also titrated by end-point dilution. Virus-containing media were clarified from cells and cell debris by centrifugation (10000 g for 10 min) and 0.2 ml of serially diluted media was used to infect fresh QT-6 cells. The presence of infectious virus in the media was detected by the RT assay. Five days post-infection the titre of the virus with PR\(^{38\text{Ser}}\) was estimated as \(10^4\)–\(10^5\) infectious units/ml while the titre of the virus with PR\(^{38\text{Thr}}\) was 10–100 times lower. Two independent experiments were carried out in duplicate.

Discussion

Cleavage of retrovirus Gag and Gag-Pol polyproteins requires an active virus PR (Crawford & Goff, 1985; Dittmar & Moelling, 1978; Katoh et al., 1985; Kramer et al., 1986; Le Grice et al., 1988; Leider et al., 1988; Lu et al., 1979; Moelling et al., 1980; Mous et al., 1988;...
Fig. 4. Proteolytic activity of native and mutated PR. (a) Cleavage sites of ASLV and HIV-1 Gag–Pol polyproteins. (b) Comparison of the cleavage rates of ASLV and HIV-1 oligopeptides by purified native (PR$^{38\text{Ser}}$) and mutated (PR$^{38\text{Thr}}$ and PR$^{39\text{AAs}}$) PRs. Protease activity was calculated as the percentage of substrate cleaved by the enzymes after 60 min incubation (average of duplicate reactions). The PR$^{39\text{AAs}}$ cleavage products were not detected by HPLC analysis and are therefore not included. The numbered synthetic peptides (SP) correspond to the cleavage sites in (a). (c) Synthetic oligopeptides homologous to the cleavage sites of ASLV and HIV-1. Oligopeptides were incubated with PR$^{38\text{Ser}}$ and PR$^{38\text{Thr}}$ and the amino acid composition of the reaction products was determined. The cleavage sites are indicated by (...).
ASLV PR mutation

reasonable to assume that PR found in mammalian retroviruses is significantly more active than its avian counterpart.

The active site of the ASLV PR contains Ser whereas PRs of mammalian retroviruses contain Thr at the equivalent position. This change could account for the difference in activity. We found that ASLV PR with Thr instead of Ser at the active site is more active than the wt enzyme, although the specificity of the mutant enzyme for both ASLV and HIV-1 substrates remained unchanged. It could be argued that the increased activity of PR containing the Thr substitution is caused either by efficient refolding during purification or by factors unrelated to the activity of the enzyme. This seems rather unlikely, since the soluble fraction of bacterial extracts

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expressing wt and mutated PRs clearly shows that PR\textsuperscript{38Thr} hydrolyses synthetic peptides homologous to ASLV and HIV-1 more rapidly than wt PR.

It is interesting that replacement of Ser with Thr at position 38 of the viral PR is sufficient to reduce the production of infectious virus and RT activity in the medium. Differences in transfection efficiency cannot unequivocally be the reason for these differences because identical amounts of plasmid DNA, which differed by only 3 bases, were used to transfect parallel QT-6 cell cultures. These results therefore suggest that a mutation responsible for increased activity of PR decreases the production of infectious virus and RT activity in the medium. However, these results cannot clarify whether less active RT per virion is present or whether normal amounts are produced but with lower specific activity.

The reduced activity of PR\textsuperscript{38Thr} in processing virus precursors expressed in avian cells is in contrast to the results obtained in bacterial cells and in assays \textit{in vitro}. The presence of unprocessed Gag polyprotein in particles containing PR\textsuperscript{38Thr} could be due to PR release from the precursor(s) or inactivation of PR prior to virus assembly. It is also possible that in an avian cell where particles can assemble, the mutation leads to improper folding so that the active site is less accessible, while the bacterial extracts lack a cellular factor(s) required for proper folding and therefore the enzyme is active. Sedlacek \textit{et al.} (1993) demonstrated that mutated PR (at positions 100, 104, 105, 106 and 107) with increased proteolytic activity caused the production of morphologically altered particles which retained unimpaired infectivity. Other mutations in the catalytic region of PR produced inactive enzyme. In addition, mutating the adjacent conserved Ala at position 40 to Thr also produced an inactive enzyme, but replacing this Ala residue with Ser only reduced the rate of catalysis (Grinde \textit{et al.}, 1992).

The conversion of a Ser to a Thr codon requires the replacement of a single nucleotide. Since retroviruses have a high mutation rate (Leider \textit{et al.}, 1988), it seems likely that with evolution over time the conversion of Ser to Thr in ASLV PR might have taken place, if this mutation could have provided a selective advantage. Presumably it is disadvantageous to the virus to produce PR with either too much or too little activity. Appropriate regulation can be obtained by controlling the amount of PR synthesized either by the frameshift mechanism or by modulating its specific activity. Using oligopeptides homologous to the putative cleavage sites in the ASLV and HIV-1 Gag–Pol precursors as substrates, we demonstrated that PR\textsuperscript{38Ser} and PR\textsuperscript{38Thr} have similar substrate specificities. Thus, the specific activity of the enzyme is a crucial factor for allowing release of active particles. PR\textsuperscript{38Thr} may be active before virion assembly and might therefore interfere with the maturation of the particles. This explanation is based on studies carried out by Burstein \textit{et al.} (1991) and Krausslich (1991) who showed that processing of precursors containing two linked PR molecules was completed entirely within the cell, without the release of virus particles. Together, these results suggest that although PR\textsuperscript{38Thr} produced in bacterial cells preserves the characteristics of PR\textsuperscript{38Ser}, fine regulation of PR activity is a crucial factor in the optimization of virion maturation.

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**References**


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