Human Immunodeficiency Virus Type 1 Proteinase Is Rapidly and Efficiently Inactivated in Human Plasma by $\alpha_2$-Macroglobulin

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Human plasma impairs the activity of the human immunodeficiency virus (HIV-1) proteinase to cleave the HIV-1 gag-polyprotein precursor. The inhibition is due to the entrapment of the proteinase by plasma $\alpha_2$-macroglobulin ($\alpha_2$M). In methylamine-treated plasma, where $\alpha_2$M is inactivated, HIV proteinase is not blocked. The interaction of $\alpha_2$M and HIV proteinase resulting in covalent complexes of proteinase and $\alpha_2$M was demonstrated by immunoblotting with antisera either to $\alpha_2$M or to the HIV proteinase. We suggest if HIV-1 proteinase would be released in vivo from infected patients' cells, $\alpha_2$M entrapment may prevent or minimize a conceivable cleavage of extracellular matrix or plasma proteins by the HIV-1 enzyme.

Key words: Aspartic proteinase / Proteinase inhibitors / Macroglobulin / Retroviral proteinase.

The aspartic proteinase of the human immunodeficiency virus (HIV PR) type 1 is responsible for the maturation to infectious virus by processing the viral gag and gag-pol precursor proteins. The viral proteolytic activity in infected host-cells might, however, not be restricted to the processing of viral proteins. Purified recombinant HIV proteinase is capable of cleaving in vitro a number of host cell proteins (particular cytoskeletal ones) (Shoeman et al., 1991; Oswald and von der Helm, 1991; Poorman et al., 1991; Riviere et al., 1991; Tomasselli et al., 1991). The cleavage of vimentin, myosin (Lindhofer et al., 1993) and actin (Adams et al., 1992) has even been shown in HIV infected cultured cells to be due to the HIV PR activity, it can be prevented by HIV PR inhibitors. HIV PR activity is also conceivable at the surface of infected cells or extracellularly, taking place by release of the proteinase from damaged or dying HIV-infected cells. Antibodies to HIV PR, found in blood of more than one third of HIV-infected patients (Boucher et al., 1989), support the probability of cell surface exposure of viral proteinase to the immunosystem.

Fig. 1 Inhibition of HIV PR by Human Plasma. Immuno blot of Gag-Cleavage Assay.

2.5 μl of highly purified recombinant HIV PR (Helm et al., 1994) (9.5 μg HIV PR/ml = 0.43 μM in 25 mM MES, pH 6.0, 0.2 μM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 5 mg/ml BSA) (lanes 2–4) or MES-buffer alone (lane 1) were incubated for 30 minutes at 37 °C with 2.5 μl of either untreated normal human plasma (containing 2.2 μM of active $\alpha_2$M, E:$\alpha_2$M molar ratio 1:5), or methylamine-treated human plasma (no $\alpha_2$M activity detected), or BSA (10 mg/ml in PBS). Thereafter 30 μl supernatant of an Escherichia coli lysate, containing recombinant HIV-1 gag polyprotein (Seelmeier et al., 1988) (in 50 mM MES, pH 6.0, 1 mM DTT, 1 mM EDTA, 0.1% Tween 20, 1 μg/ml leupeptin and 1 mM PMSF) were added to the samples, followed by incubation for 25 minutes. Reaction mixtures were boiled in gel-loading buffer, run in 12% reducing SDS-polyacrylamide gels (Laemmli, 1970), blotted on nitrocellulose filters, stained with affinity purified sheep antibodies, recognizing gag-p24, gag-p55 and intermediate precursors (Seromed).
site of α₂M (Athauda et al., 1993) is in agreement with the known mechanisms of α₂M action on other proteinases (Sottrup-Jensen, 1989; Borth, 1992; Salvesen and Enghild, 1993). As a result of this action the in vitro cleavage of viral gag-precursor protein is impaired, apparently because polyprotein substrate has no access to proteinase entrapped in the α₂M 'cage'.

Here, we tried to mimic the physiological event of a possible release of active HIV PR from infected cells into plasma. Purified recombinant HIV PR was incubated for 30 minutes at 37 °C with normal human plasma or methylamine treated plasma prior to assaying the cleavage of recombinant HIV-1 gag protein precursor as substrate (here described as ‘gag-cleavage assay’) and followed by immunoblot analysis of the reaction mixture.

HIV PR, treated with normal plasma, was inhibited (Figure 1, line 2); the precursor gag-p55 and intermediate gag-precursors (which artificially arose by partial unspecific proteolysis during preparation of the recombinant lysate) remained as uncleaved, as they did in the control gag-lysate without HIV PR, mock-treated with plasma (lane 1). HIV PR, preincubated with either methylamine-treated plasma (lane 3) or, as a control, with BSA solution (BSA was used to prevent proteinase self-degradation) (lane 4), processed the gag-p55 and most of the intermediates precursors correctly (the remaining ‘uncleaved’ bands represented lysate proteins unspecifically reacting with antiserum). As methylamine is known to destroy the α₂M inhibitor capacity via inactivation of its thiol-ester active site (Barret, 1981), we assume that the observed inhibition of HIV PR by plasma is due to α₂M.

To confirm this, we tried to demonstrate α₂M:HIV PR as covalent complexes in plasma (Figure 2) by trapping them in immunoblots. HIV PR incubated with plasma was run in reducing SDS-PAGE in parallel, blotted to two nitrocellulose filters, one of them stained with antiserum to HIV PR and the other one stained with antiserum to α₂M. Antiserum to proteinase recognized two new intensive bands of molecular weights 110 and 120 kDa (Figure 2A, lane 2) much higher than the 11 kDa of HIV PR itself (seen in lane 3). Antiserum to α₂M recognized three new bands, smaller than the 185 kDa α₂M monomer of Figure B, lane 1: two of them matching in size to the 110/120 kDa bands, recognized by antibodies to HIV PR, and one additional 95 kDa band (lane 2). The new bands were much less intensive when plasma and HIV PR had been preincubated with the specific low molecular weight inhibitor of HIV PR, Ro 31-8959, (Figures A and B, lanes 4). After the methylamine-treated plasma preincubation the 110/120 kDa bands were not detected, although the 95 kDa band was clearly pre-

**Fig. 2 Detection of HIV PR-α₂M Complex in Human Plasma by Immunoblotting.**

3 μl of untreated plasma or BSA or methylamine-treated plasma (see legend Figure 1) were incubated for 30 minutes at 37 °C with 2 μl of either HIV PR [68 μg/ml = 3.1 μM (determined by active site titration) in 50 mM MES, pH 6.0, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA] (E:α₂M molar ratio 1:1) or MES-buffer in the presence or absence of HIV PR inhibitor, Ro 31-8959 (Roberts et al., 1990), at a concentration of 3 μM (E:1 molar ratio 1:2.4). Thereafter samples were boiled in gel loading buffer and aliquotes were run in 6—16% gradient SDS-polyacrylamide gel (Laemmli, 1970) (1.5 μl in A and 0.05 μl in B) and blotted to nitrocellulose filters, which were stained with: A, polyclonal rabbit antiserum to the HIV PR (kind gift of B. Korant, Du Pont Merck, USA); B, polyclonal goat antiserum to human α₂M (Nordic Immunological Laboratories). Lanes: 1, untreated plasma; 2, untreated plasma and HIV PR; 3, no plasma, but BSA and HIV PR; 4, untreated plasma and HIV PR with HIV PR inhibitor Ro 31-8959; 5, methylamine-treated plasma and HIV PR; 6, methylamine-treated plasma, HIV PR and inhibitor Ro 31-8959.
sent (Figures 2A and B, lanes 5). The unspecific bands (about 60 kDa), detectable with antiserum to HIV PR in figure A, resulted from non-specific stain of inherent large amounts of plasma albumin (40 μg pro lane).

The detected 110/120 kDa fragments, reacting with antiseras to both HIV PR and α2M, are likely to represent HIV PR bound to the C-terminal fragment of cleaved α2M in a covalent way, since they were not removed by boiling in DTT and SDS. The binding is probably through thiol-ester site, which is located downstream the HIV PR cleavage site. The rationale for two such bands instead of one expected band is not yet clear. The 95 kDa fragment, reacting in our study only with antiserum to α2M, is likely to represent one of the cleavage fragments of the α2M molecule, non-complexed to the HIV PR. The interaction of purified α2M and HIV PR was reported earlier to yield fragments of the same size (Meier et al., 1991; Athauda et al., 1993). Preincubation with a specific inhibitor of HIV PR prevented significantly proteolytic activity and thus cleavage of α2M and consequently covalent complexing (Figures A and B, lanes 4). In methylamine-treated plasma α2M could still be cleaved (see 95 kDa band in lane 5, Figure B), because HIV PR was active, but no covalent bands between α2M fragments and HIV PR were formed because the thiol-ester site was inactivated (Figure A, lane 5).

Proteinases trapped within the α2M molecule ought not to be recognized by humoral antibodies (Barret, 1981), because immunoglobulins cannot reach into α2M 'cage'. Treatment by SDS and DTT as prerequisite to SDS-PAGE and immunoblot broke, however, the 'cage' structure, but keeping the covalent bonds of HIV-PR:α2M complexes and thus rendered them immunoreactive, as demonstrated by our results.

The interaction of HIV PR with the plasma α2M, described here, was rapid and efficient. In separate experiments (not shown) incubation of HIV PR with normal plasma for as short as one minute was sufficient to prevent most of the subsequent gag-precursor cleavage. Even when HIV PR, plasma and gag-precursor lysate were mixed simultaneously still a partial inhibition of processing took place. Hence, we assume, that α2M might play a protective role against a conceivably HIV proteolytic activity at surfaces of HIV infected cells or in human plasma, in analogy to the role α2M plays in the protection of proteins against the action of bacterial proteinases (Miyata et al., 1981; Miyoshi and Shinoda, 1989).

Preliminary results of analogous experiments but carried out with the recombinant proteinase of the human T-cell leukemia (type 1) virus, (HTLV), seem to be different from the HIV PR results. HTLV proteinase cleaves α2M in plasma very poorly, and we have not yet evidence whether or not HTLV proteinase can be entrapped by α2M.

Finally, Chu et al., reported (1994), that delivery of antigen by α2M complexing enhances antibody formation. An entrapment of HIV PR by α2M in human plasma might thus aid the formation of antibodies against the proteinase, and explain the high proportion of proteinase antibodies found in HIV infected patients sera (Boucher et al., 1989).

Acknowledgments
We are grateful to Dr. S. Gruenik (PRI-NCI, Frederick, MD, USA) for determination of the HIV PR concentration by the active site titration, to Dr. P. Nawratil (University of Munich) for providing human plasma, to S. Seelmeir for preparation of recombinant gag-lysate, to Drs. H. Fritz and H. Nitschko (University of Munich) for discussions and critical reading of the manuscript and to Roche, UK, for providing Ro 31-8959. The work was supported by the HIV Forschungsprojekt, BMFT.

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Received June 30, 1994; accepted August 5, 1994
Short Communication

Two-Chain Bacteriorhodopsin Synthesized by Schizosaccharomyces pombe

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Bacteriorhodopsin (BR), the light-driven proton pump of Halobacterium salinarium purple membrane, was produced in functional form as a two-chain protein by simultaneous expression in the fission yeast Schizosaccharomyces pombe of two separate structural genes, one coding for an aminoterminal BR fragment encompassing the first two transmembrane helices of BR, the other coding for the remainder of the protein. The fragments assemble spontaneously in vivo to yield functional BR which can be purified by immobilized metal ion affinity chromatography.

Key words: Halobacterium salinarium / Heterologous gene expression / Protein engineering / Membrane design / Light-driven proton pump / Retinal protein.

Bacteriorhodopsin (BR) is the only protein component of the purple membrane of Halobacterium salinarium within which it forms two-dimensionally ordered arrays and serves as a light-driven proton pump (Oesterhelt et al., 1992; Krebs and Khorana, 1993). It consists of seven membrane-spanning helices (marked A–G in Figure 1), connected by short loops (Henderson et al., 1990). Retinal is attached by a Schiff base linkage to the ε-amino group of lysine residue #216, located in helix G. Replacement of one or several extra-membrane parts of bacteriorhodopsin by exogenous polypeptide modules could provide access to multifunctional membranes for various applications. The attractivity of such an approach stems from (i) ready detection of correct overall folding of the hybrid proteins by monitoring absorption at 550 nm (purple colour), (ii) possibility of controlling the steric arrangements of functional modules relative to one another and, (iii) possibility of combining functions carried by exogenous modules with light-driven proton pumping of BR itself.

We have replaced various loops of BR by modules such as a 13 amino acids long linear epitope of Sendai virus L protein (Teufel et al., 1993), a DNA binding domain of

Fig. 1 Membrane Insertion Topology of Bacteriorhodopsin and Two-Chain Bacteriorhodopsin.

Upper panel: Structure of bacteriorhodopsin (schematic). The shaded boxes marked A–G represent membrane-spanning α-helices according to the BR structure described by Henderson et al. (1990). Numbers refer to residue positions in natural BR [note the two additional amino acid residues at the N- and the C-terminals present in BR produced by gene expression in S. pombe (Teufel et al., 1993)]. In the cell membrane of Halobacterium salinarium, the upper three extra-membrane loops, together with the C-terminus, face the archetal cytoplasm.

Lower panel: Pausible structure of two-chain bacteriorhodopsin (schematic). Fragment AB (light shading) is 88 amino acid residues long and consists of the amino-terminal part of BR, followed by an aspartic acid residue and a run of six histidine residues. The Sendai epitope was introduced for immunochemical detection of fragment AB, the oligo-histidine runs for purification of both fragments by IMAC (see Figure 3).
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35 amino acids (M. Pompejus and H.-J. Fritz, unpublished) and a protease inhibitor domain of 58 amino acids (J. Hennecke and H.-J. Fritz, unpublished). In all these cases, correct overall folding of the hybrid protein and full BR activity was observed after the corresponding hybrid genes had been expressed in Schizosaccharomyces pombe and the proteins isolated from total yeast membranes.

Straight loop replacements, however, impose a number of restrictions on the choice of polypeptide modules to be inserted, most notable with respect to the distance between their N- and C-termini which has to fit the distance between two points of emergence from the membrane of the BR polypeptide chain. Additional flexibility for membrane design would be gained if functional BR could be produced as a two-chain protein and the additional chain termini used as points of fusion with exogenous polypeptide modules (compare Figure 1). Encouragement to apply such strategy came from earlier findings that functional BR can be assembled in vitro from polypeptide fragments, namely from AB and C–G (Huang et al., 1981; Popot et al., 1987), from A and B and C–G (Kahn and Engelmann, 1992) or from A–E and FG (Liao et al., 1983; Sigrist et al., 1988). With the present paper we show (i) that fragments AB and C–G (Figure 1, lower panel) can be produced in S. pombe by simultaneous expression of two separate structural genes,

Fig. 2 Construction of Expression Vector pOBS2-25 for Production of Two-Chain Bacteriorhodopsin in S. pombe. Expression vector pOBS2-25 was derived from previously described yeast vectors pREP1 (Maundrell, 1993) and pEVP11 (Russel et al., 1989). It consists of the following components. (i) Control regions of DNA replication from the genomes of the E. coli colE1 plasmid ('ori'), filamentous phage M13 ('M13') and S. pombe ('ars1'). (ii) Selectable markers for plasmid maintenance in E. coli ('bla', gene coding for β-lactamase) and in S. pombe ('Leu2', gene coding for β-isopropylmalate dehydrogenase). (iii) Expression cassettes for two fragments of the synthetic bacterio-opsin gene bos (Pompejus et al., 1993). The rightward transcription cassette consists of Pnmt, the promoter of the S. pombe nmt1 gene (Maundrell, 1989), followed by that part of the bos gene coding for BR fragment AB as shown in Figure 1 (lower panel) and concluded by a transcription terminator Tnmt (Maundrell, 1989). The leftward transcription cassette consists of Padh, the promoter of the S. pombe adh gene (Russel and Hall, 1983), followed by that part of the bos6H gene (bos gene including six histidine codons at the 3′-end; Pompejus et al., unpublished) coding for BR fragment C–G as shown in Figure 1 (lower panel) and concluded by a bona fide transcription terminator, Tpadh, a ca. 800 bp DNA fragment taken from the promoter-distal side of the multiple cloning region of pEVP11 (Russel et al., 1989).

Vector pOBS2-25 was constructed from pREPBS2-205 and pEVPBS2-215 by the following sequence of cloning steps. Vector pREPBS2-205 was digested with BglII and SmaI; protruding ends were made blunt and religated (loss of gene coding for fragment C–G). The resulting vector was linearized with PstI, protruding ends were made blunt. This fragment was cloned into the purified short SfiI/XbaI fragment of pEVPBS2-215, also made blunt.

Vector pREPBS2-205 was constructed the following way. The BamHI/Xhol fragment of the bosSEN-B/C gene (Teufel et al., 1993) was cloned into pMaSlgAbos6H (Pompejus et al., unpublished). The resulting vector pMaSlgAbosSEN-B/C-H harbours a bosSEN-B/C gene extended at its 3′-end by 6 additional histidine codons. The stretch of bosSEN-B/C-H DNA coding for the extra-membrane loop connecting helices B and C was changed as follows. Six histidine codons and two stop codons were inserted downstream of the sequence coding for the Sendai epitope, together with one restriction site each for SfiI, BglII and NdeI. Oligonucleotide-directed mutagenesis (Kunkel et al., 1987) was used employing a mutagenic primer with the following sequence: 5′-GTAGATAGGATTCTGCATATGTAGATCTGGCCGACTTGGCCCTATTAGTGATGGTGATGGTGATGTGATGAATCGTATGG-3′. The BamHI/Xhol fragment of the resulting vector (pMaSlgABS2-205) was cloned into pREP1bos6H (Pompejus et al., unpublished).

Vector pEVBS2-215 was constructed the following way. The S. pombe adh promoter of pEV11 was cloned into pGEM-11Zf(+) (Promega) as a SalI/BamHI fragment from where it was isolated as a SfiI/BamHI fragment. The large BglII/SfiI fragment of pMaSlgABS2-205 was prepared and the fragments were ligated to pMaSlgABS2-215, thereby destroying the compatible BamHI and SfiI sites. The ca. 1.5 kb BamHI/NorI fragment of pMaSlgABS2-215 was cloned into pEVBS6H (Pompejus et al., unpublished; 2 μ: origin of replication of Saccharomyces cerevisiae 2 μ plasmid).
that these fragments combine spontaneously in vivo to yield functional two-chain BR and,
that two-chain BR can be purified by immobilized metal ion affinity chromatography (IMAC).

Expression vector pOBS2-25 harbouring separate structural genes coding for BR fragments AB and C-G was constructed as indicated in Figure 2. Transcription of the two fragments is controlled by the \( P_\text{prom} \) and, respectively, the \( P_\text{adh} \) promoter. \( S. \text{pombe} \), transformed with pOBS2-25, was grown as described in Figure 3, purple tinge of yeast cells indicating an intact BR chromophore and hence correct assembly of BR in vivo. Separate expression of the individual gene fragments in \( S. \text{pombe} \), while leading to accumulation of the respective protein products, did not result in colouration of the producing cells; purified individual protein fragments also were colourless (data not shown).

Yeast crude membrane fraction was isolated and two-chain BR purified by IMAC (Figure 3). The purple colour persisted throughout the entire purification procedure; two-chain BR apparently resists dissociation by the detergents used. The yield of fragment AB conspicuously exceeds that of fragment C-G, probably due to greater strength of the \( P_\text{prom} \) promoter (Basi et al., 1993). Identities of fragments AB and C-G were confirmed by their recognition by the Sendai and, respectively, the BR2 monoclonal antibodies – both in crude mixtures and after chromatographic purification (Figure 3).

The UV/VIS spectrum of partially purified two-chain BR exhibits the characteristic BR peak at around 550 nm (in 1% octyl glucoside). Judging from the absorption at 550 nm (with an assumed \( \varepsilon_\text{max} \) of 55 000 M\(^{-1}\) cm\(^{-1}\); Dencher and Heyn, 1978), about 50 \( \mu \)g correctly folded two-chain BR were obtained from one liter \( S. \text{pombe} \) culture. An absorption ratio [280 nm]/[550 nm] of ca. 5.5, compared to a ratio of ca. 2 for homogeneous BR, attests to good but not ideal purification; note that with the production/purification scheme applied, there necessarily has to be an excess of fragment AB which does not contribute to the 550 nm chromophore. Two-chain BR pumps protons out of reconstituted liposomes into the bulk water phase (Figure 4), as was observed earlier with BR and several other BR variants produced in \( S. \text{pombe} \) (Teufel et al., 1993).

A few examples have been described of dissecting integral membrane proteins by simultaneous expression of two gene fragments to yield functional two-chain derivatives, in greatest detail that of \( \text{Escherichia coli} \) Lac permease (Wrubel et al., 1990; Bibi and Kaback, 1990; Wrubel et al., 1994; Zen et al., 1994). Genetic complementation of \( \text{lacY} \) mutants to a Lac\(^+\) phenotype was demonstrated but the products were not characterized biochemically. Similar results were obtained with two mammalian receptors of the seven transmembrane domains class, \( \beta \)-adrenergic receptor (Kobilka et al., 1988) and muscarinic acetyl cholin receptor (Maggio et al., 1993). Split receptors were functional in assays performed on whole cells and unfractionated membrane isolates. Studies of this kind may not only give more general access to membranes with
engineered properties (as outlined above), but may also be useful for testing certain working hypotheses of biosynthesis and structural chemistry of membrane proteins such as, in particular, the ‘two stage model’ (Popot and Engelman, 1990, Popot and de Vitry, 1990) of membrane protein folding.

Acknowledgements

This work was supported by the German Minister for Research and Technology (BMFT). We thank H. Einberger, P.H. Hofschneider, K. May, W. Neubert and D. Oesterhelt for their gift of monoclonal antibodies. M. Pompejus was recipient of a Kekulé predoctoral fellowship provided by ‘Fonds der chemischen Industrie’.

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*Edited by K. Nagai*
*Received August 3, 1994; accepted August 30, 1994*