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Characterization of the Immunoglobulin A Protease of *Ureaplasma urealyticum*

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***Ureaplasma urealyticum* strains of all serotypes express a specific human immunoglobulin A1 protease that cleaves immunoglobulin A1 to produce intact Fab and Fc fragments. The use of a variety of inhibitors suggests that the enzyme is a serine protease. N-terminal sequencing of the Fc digestion product showed that the enzyme cleaves between the proline and threonine residues 235 and 236 in the hinge region of the heavy chain of immunoglobulin A1.**

Immunoglobulin A (IgA) is the main immunoglobulin present at mucosal surfaces. Several pathogenic organisms, including *Ureaplasma urealyticum*, in which adhesion to a mucosal surface may be the first step in pathogenesis have been shown to produce human IgA1-specific proteases that cleave IgA1 into intact Fab and Fc fragments (8, 9, 14, 16). Generally, these enzymes cleave the heavy chain at proline-threonine or proline-serine bonds within the hinge region (7).

In this report we describe initial studies of the IgA1 protease of *U. urealyticum*.

Ureaplasma strains of serotypes 1 (T7), 4 (11860), 6 (12253), and 8 (T960) were gifts from D. Taylor-Robinson (Clinical Research Center, Harrow, United Kingdom); the serotype 7 strain (27819) was obtained from the American Type Culture Collection, and strains of serotypes 2 (T23), 3 (DKF 3), 5 (NIH 5), 9 (9-Vancouver), 10 (10-Western), 11 (11-JsL 2), 12 (12-JsL 5), 13 (13-JsL 6), and 14 (14-JsL 11) were gifts from J. Robertson (University of Alberta, Edmonton, Canada). All ureaplasmas were cultured and harvested as described previously (21). Pellets were washed three times with Dulbecco phosphate-buffered saline A (PBS) (4), resuspended in PBS at 2 mg of protein · ml^{-1} (13), and used fresh or after storage at −70°C as a source of crude protease. As substrates for the crude enzyme, human colostral secretory IgA (sIgA; Sigma), purified human IgA1 (Calbiochem), or purified human IgA2 (gift from M. Kerr, Department of Pathology, Ninewells Hospital) was used.

To demonstrate protease activity, generally, 5 μl of ureaplasma suspension was incubated (16 h, 37°C) with 15 μl of sIgA (1 mg of protein · ml^{-1} in PBS); as a control, 15 μl of sIgA in 5 μl of PBS was treated similarly. After incubation, the suspension was centrifuged (12,000 × *g*, 5 min) and the supernatants were assayed for IgA1 digestion products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% (wt/vol) polyacrylamide gels at a constant voltage of 150 V. Samples were denatured before loading (21); after electrophoresis, gels were fixed for 1 h in 10% (wt/vol) trichloroacetic acid–40% (vol/vol) methanol, stained for 4 h in 0.3% (wt/vol) PAGE blue 83 (Sigma)–46% (vol/vol) methanol–7.5% (vol/vol) acetic acid, and destained in 7.5% (vol/vol) acetic acid–25% (vol/vol) methanol. Alternatively, electrophoretic transfer of the electropherograms to nitrocellulose sheet was followed by blocking in 10% (wt/vol) Marvel (Cadbury) in PBS. After the blocked nitrocellulose sheets were rinsed with PBS, they were incubated (overnight, 20°C) with a 1/1,000 dilution of sheep anti-human IgA (Scottish Antibody Production Unit) in 5% (wt/vol) Marvel in PBS, washed (once with PBS [10 min], twice with PBS–0.5% [vol/vol] Nonidet P-40 [Sigma] [10 min for each wash], and once with PBS [10 min]), and then incubated (3 h, 20°C) with a 1/500 dilution of peroxidase-linked donkey anti-sheep antibody (Scottish Antibody Production Unit) in 5% (wt/vol) Marvel in PBS. After the nitrocellulose sheet was washed again as described above, it was developed by incubation with a substrate (22). For other assays, IgA1 was radiolabeled with ^{125}I by adding 0.5 mCi of ^{125}I ($Na^{125}I$, 100 mCi · ml^{-1}; Amersham International) to 20 μl of IgA1 (1 mg of protein · ml^{-1} in PBS). The reaction was initiated at 20°C by adding 20 μl of chloramine T in PBS (0.2 mg · ml^{-1}) and terminated after 2 min by adding 20 μl of sodium metabisulfite in PBS (0.3 mg · ml^{-1}) and then 40 μl of PBS. Unbound label was removed by centrifugation (300 × *g*, 3 min) of the reactant through a 1-ml column of medium Sephadex G50 (Pharmacia). Labeled protein was diluted to 1 ml (0.02 mg of protein · ml^{-1}, ~10^6 cpm · ml^{-1}). Proteolytic digestions were carried out as described above but with 3 μl of ^{125}I-labeled IgA1 and the volume difference made up with PBS. After SDS-PAGE, digestion products were detected by fixing the gels (as described above), drying them, and then subjecting them to autoradiography overnight at −70°C with X-ray film (Fuji-RX) and a Philips fast tungstate intensifying screen.

Our initial studies confirmed that human colostral IgA is partially digested to produce intact Fab_α and Fc_α fragments of 28.5 and 33 kDa and that all 14 serotypes of *U. urealyticum* express the enzyme (Fig. 1), as reported previously (9, 10, 20). Furthermore, we confirmed that human IgA2 (with a deletion in the hinge region) was not a substrate (data not shown). It was apparent that the enzyme activity could only be detected in either suspensions of ureaplasma cells or soluble extracts (partial cellular solubilization in PBS–0.5% [vol/vol] Nonidet P-40). This suggested that the enzyme was membrane associated and possibly surface expressed. Since this was contrary to a previous report (9) that suggested the release of a soluble enzyme, as is the case with other bacterial IgA proteases, we attempted to repeat that procedure. Spent medium after cell harvest (centrifugation at 30,000 × *g* for 20 min) was concentrated 20-fold by positive

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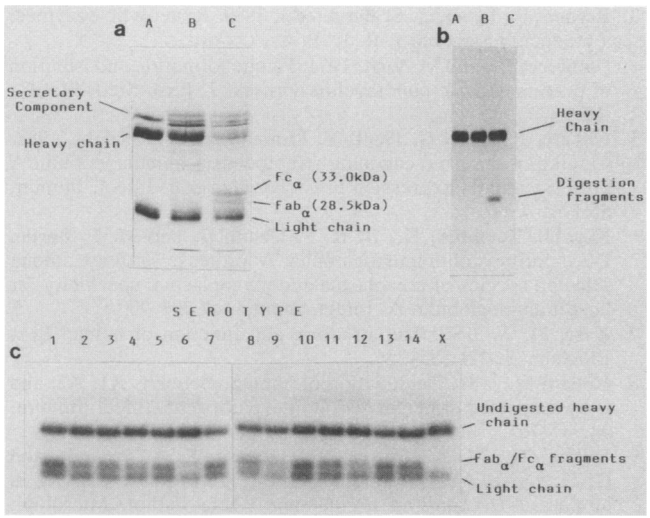

FIG. 1. (a) Digestion of human colostral secretory IgA by *U. urealyticum* serotype 8 IgA1 protease. Lanes: A, control (no added ureaplasma suspension); B, 20 s after addition of ureaplasma suspension; C, detection of Fab$_\alpha$ and Fc$_\alpha$ digestion products after incubation with ureaplasma suspension. Note: other bands in lanes B and C are due to ureaplasmal proteins. The 12.5% (wt/vol) polyacrylamide gel was stained with PAGE blue 83. (b) Immunoblot with ^{125}I-labeled human IgA1 as the substrate, showing appearance of digestion products after incubation. Lanes: A, control (no added ureaplasma suspension); B, 20 s after addition of ureaplasma suspension; C, detection of comigrating digestion products after incubation with ureaplasma suspension. The immunoblot was probed with sheep anti-human IgA heavy-chain antibody and then peroxidase-linked donkey anti-sheep antibody. (c) Autoradiogram displaying IgA1 protease activity in the 14 serotypes (lanes 1 through 14) of *U. urealyticum*. Each assay performed with ~3 μl of labeled substrate and equal amounts of the individual serotypes (based on the concentration of protein in the suspensions). X, control with labeled substrate alone.

pressure ultrafiltration with an Amicon P10 membrane. The concentrate (15 μl) was assayed for protease activity by incubation (16 h, 37°C) with 15 μl of sIgA (4 mg · ml^{-1} in PBS). The assays were subjected to SDS-PAGE for detection of digestion products. The presence of so many medium components in the concentrated samples so distorted the electropherograms that detection of specific digestion products was not possible. Because of this, immunoelectrophoresis was not attempted. Further studies were carried out in an attempt to detect the enzyme in the medium. With ^{125}I-labeled IgA1 (3 μl), spent medium after cell harvest was assayed for protease activity as described above, except that 17 μl of spent medium replaced 5 μl of cell suspension. After SDS-PAGE and autoradiography, no digestion products were detected. In addition, to minimize contamination with medium components, 50 ml of centrifuged spent medium was subjected to ammonium sulfate precipitation with fractions taken at 10% saturation stages from 20 to 100%. The precipitates were centrifuged (12,000 × g, 10 min, 20°C), suspended in 1 ml of PBS, dialyzed overnight against PBS (4°C), and then assayed for IgA protease activity with 60 μl of dialysate and 5 μl of ^{125}I-labeled IgA1. With this technique, again no evidence was found for the enzyme being released from the cells, although there was still substantial interference in the fractions from medium components. It is interesting to note that genes coding for other bacterial IgA proteases (17, 18) appear to have three domains, a helper domain, a functional domain, and an export domain, and it may be that the last is not present in the ureaplasma enzyme.

TABLE 1. Inhibition of the ureaplasma IgA1 protease by serine protease group-specific inhibitors

Inhibitor	Inhibitor concn (μM)	% Inhibition
3-4-Dichloroisocoumarin in DMSO[a]	10.0	20
	50.0	40
	100.0	100
Di-isopropylfluorophosphate in propan-2-ol	1.0	0
	10.0	90
	100.0	100
	1,000.0	100
Di-isopropylfluorophosphate in propan-2-ol control[b]	1.0	0
	10.0	40
	100.0	85
	1,000.0	100

[a] DMSO, dimethyl sulfoxide.
[b] Positive control with trypsin (serine protease) at 0.1 mg · ml^{-1} in a standard asocasein assay (12).

In an attempt to define the nature of the protease more precisely, protease inhibitors were assayed for their effect on the enzyme activity by incubation at appropriate concentrations in the relevant solvents (3). Crude protease preparations (5 μl in 25 μl of PBS) were incubated with 5 μl of inhibitor (30 min, 37°C), and 15 μl of IgA was added (labeled or unlabeled); after further incubation (16 h, 37°C) and immunoblotting or autoradiography, the extent of enzyme inhibition was determined. Control incubations with inhibitor solvent alone were carried out where appropriate. No inhibition of enzyme was seen with cysteine protease inhibitors (10.0 to 100.0 μM aqueous iodoacetamide, 0.01 to 0.1 mg of cystatin [egg white or human] per ml in 10 mM Tris [pH 8]–50% [vol/vol] glycerol, 1.0 to 10.0 μM aqueous E64, 8.0 to 18.0 mM aqueous *N*-ethylmaleimide), with aspartyl protease inhibitors (1.0 μM pepstatin in methanol, 1.0 μM bromophenacyl bromide in methanol), or with metalloprotease inhibitors (5.0 mM phenanthroline in methanol, 5.0 to 166.0 mM aqueous EDTA). Stability of the enzyme in EDTA had been reported previously (20). Results obtained with known serine protease inhibitors were variable. No inhibition was achieved with the more restrictive protease inhibitors (100.0 to 500.0 μM aqueous aprotenin, 10.0 to 100.0 μM chymostatin in dimethyl sulfoxide, 100.0 mM tosyl phenylalanyl chloromethyl ketone in methanol, 100.0 mM aqueous tosyl lysyl chloromethyl ketone [TLCK], 100.0 mM aqueous leupeptin). However, significant inhibition by serine protease group inhibitors (Table 1) suggests that the *U. urealyticum* IgA1 protease does belong to the serine protease class.

To determine the IgA1 cleavage site, enzyme digestion was performed as described above with 90 μl of colostral IgA and 30 μl of ureaplasma suspension (serotype 8) made to a total volume of 150 μl with PBS. To block and to enable detection of free sulfhydryl groups (cysteine residues) in the Fc$_\alpha$ digestion product, after centrifugation (12,000 × *g*, 5 min, 4°C) the supernatant was dried onto the surface of an Eppendorf tube by rotary evaporation and then pyridylethylated as described by Amons (1). The reaction product was treated with gel electrophoresis sample preparation buffer containing 1% (wt/vol) SDS (11), and run as three tracks on SDS-PAGE (12.5% [wt/vol] polyacrylamide) with a Mighty

Small II apparatus (Hoefer Scientific Instruments Ltd.) as described in Applied Biosystems User Bulletin no. 25. In this instance, piperazine diacrylamide was used as gel cross-linker instead if bisacrylamide. The gel was electroblotted onto an Immobilon P membrane (Millipore): the gel was stained with 0.1% (wt/vol) Coomassie blue (Serva) in 50% aqueous methanol, and the Fc fragments were excised with a scalpel. The N terminal amino acids of the excised band were sequenced by automatic Edman degradation with an Applied Biosystems model 477A pulsed-liquid sequencer equipped with a 120A on-line ptH analyzer and the manufacturer's BLOTT 1 cycle. Under the conditions used before N-terminal amino acid sequencing, the Fab_α and Fc_α fragments migrated as two close bands (Fig. 1). Although care was taken to excise only the Fc_α fragment, part of the Fab_α fragment could also have been excised. However, the N-terminal amino acid of the latter is glutamic acid (19); under the conditions used for sequencing, this amino acid readily cyclizes to pyrrolidine carboxylic acid and thus becomes unavailable to the usual N-terminal reagents (15). Thus, a single N-terminal sequence was obtained for the Fc_α fragment alone. This was reproducible and was -Thr-Pro-Ser-Pro-Ser-Cys-Cys-His-Pro-.

The designation of the two cysteine residues was only possible after pyridyl ethylation of the Fc_α fragment, since under normal conditions of Edman degradation cysteine residues cannot be identified. By comparison with the known amino acid sequence of human IgA1 (19), the N-terminal sequence is consistent with a scission between residues 235 and 236 located in the second repeat of the octapeptide within the hinge region of the heavy chain. This was one of two previously predicted cleavage sites (6); since this region is deleted in human IgA2, the substrate specificity of the enzyme is explained. The only other bacterial IgA1 proteases with a similar cleavage site are the type 2 proteases of *Neisseria gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae* (7). Why these enzymes split this bond in only one of the repeating octapeptides remains to be elucidated. Nevertheless, it is tempting to draw attention to the similarities between the IgA proteases of *U. urealyticum* and of *N. gonorrhoeae* type 2, since both organisms may be isolated from the urogenital tract and both enzymes cut IgA1 at the same peptide bond and appear to be serine proteases (2). To what extent these enzymes may be related awaits the isolation and sequencing of the gene for the ureaplasma protease. However, it should be remembered that, whereas the IgA proteases of *Streptococcus pneumoniae* and of *S. sanguis* cleave at the same site, DNA hybridization studies have not revealed significant homology between their genes (5).

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