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Proteolysis of Bacterial Membrane Proteins by *Neisseria gonorrhoeae* Type 2 Immunoglobulin A1 Protease

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The immunoglobulin A1 (IgA1) proteases of *Neisseria gonorrhoeae* have been defined as having human IgA1 as their single permissive substrate. However, in recent years there have been reports of other proteins which are susceptible to the proteolytic activity of these enzymes. To examine the possibility that gonococcal membrane proteins are potential substrates for these enzymes, isolated outer and cytoplasmic membranes of *N. gonorrhoeae* were treated in vitro with exogenous pure IgA1 protease. Analysis of silver-stained sodium dodecyl sulfate-polyacrylamide gels of outer membranes indicated that there were two outer membrane proteins of 78 and 68 kDa which were cleaved by IgA1 protease in vitro in GCM 740 (a wild-type strain) and in two isogenic IgA1 protease-negative variants. Similar results were observed with a second gonococcal strain, F62, and its isogenic IgA1 protease-negative derivative. When GCM 740 cytoplasmic membranes were treated with protease, three minor proteins of 24.5, 23.5, and 21.5 kDa were cleaved. In addition, when outer membranes of *Escherichia coli* DH1 were treated with IgA1 protease, several proteins were hydrolyzed. While the identities of all of these proteolyzed proteins are unknown, the data presented indicate that there are several proteins found in the isolated membranes of gram-negative bacteria which are permissive in vitro substrates for gonococcal IgA1 protease.

The enzymatic activity now called immunoglobulin A1 (IgA1) protease was first described in 1973 by Mehta et al. when they demonstrated the presence of a bacterial enzyme in human stool samples which produced intact Fab_α and Fc_α fragments from human IgA (19). Since that time, several bacteria have been reported to produce enzymes with this activity and these enzymes have been the subject of several review articles (13, 21, 30). Bacteria which produce IgA1 proteases are clustered into a broad grouping of human mucosal surface pathogens. This group includes the gram-negative bacteria *Neisseria gonorrhoeae* and *Neisseria meningitidis* (31), *Haemophilus influenzae* (14, 17, 25), *Bacteroides melaninogenicus* (11), *Bacteroides asaccharolyticus* (11), and *Capnocytophaga* sp. (11). There are also several gram-positive IgA1 protease producers, such as *Streptococcus pneumoniae* (14, 17, 25), *Streptococcus sanguis* (32), *Streptococcus mitis* (35), *Streptococcus oralis* (12, 35), and *Clostridium ramosum* (6) as well as one mycoplasma, *Ureaplasma urealyticum* (36). Because many of the organisms that produce IgA1 proteases are mucosal pathogens of humans and the IgA1 proteases specifically cleave a major component of the secretory immune system, the production of IgA1 protease has been postulated to be a virulence factor for these organisms (13).

It has been reported repeatedly that this group of enzymes is specific for a single substrate, the hinge region of human IgA1 (21, 30). While a number of relevant human and animal proteins have been tested as potential substrates, the only permissive substrates for these enzymes were the IgA1 molecules from humans and higher primates. Each IgA1 protease cleaves a single peptide bond within the duplicated octet of amino acids that is present in the hinge region of human IgA1 and missing in IgA2 because of a deletion (21, 30). *N. gonorrhoeae*, as well as *N. meningitidis* and *H. influenzae*, produces at least two types of IgA1 proteases as defined by the specific peptide bond cleaved (22, 24, 26). Other of the IgA1 protease-producing species cleave within this same hinge region but at different peptide bonds (21, 30).

The enzymes from *N. gonorrhoeae* have been particularly well studied, having been cloned in several laboratories (8, 15, 23). The entire nucleotide sequence of an *iga* gene has been reported as well as a proposed model for the secretion of the gene product from the gonococcus (33). According to the model, an N-terminal leader peptide is responsible for secretion of the entire 169-kDa *iga2* gene product through the cytoplasmic membrane (CM) of the gonococcus. Next, a 45-kDa carboxyl-terminal helper region (β protein) mediates attachment to the outer membrane (OM) and forms a pore through which the proenzyme is extruded. Once exposed on the extracellular side of the OM, the proenzyme releases itself via an autoproteolytic event. Finally, in the fluid phase, IgA1 protease makes a second clip in its own sequence to release either a 12- or 15-kDa peptide (α) of unknown function (33). This report was the first description of a substrate other than human IgA1 for the gonococcal enzyme. The same research group subsequently reported that the gonococcal IgA1 protease is capable of cleaving two other human proteins, granulocyte-macrophage colony-stimulating factor and the CD.8 surface antigen of cytotoxic T lymphocytes (34). These data raise the question that there might be other unrecognized substrates for the IgA1 proteases.

We have developed procedures for the purification of both type 1 and 2 gonococcal IgA1 proteases (39). The ability to produce pure gonococcal type 2 IgA1 protease and the construction of an isogenic set of IgA1 protease-positive and -negative variants of *N. gonorrhoeae* have allowed us to examine the question of whether IgA1 proteases may be involved in the modification of the gonococcal cell surface via proteolysis of gonococcal membrane proteins and whether these enzymes may have more permissive substrates than had previously been described. We have examined the susceptibility of isolated OMs and CMs to IgA1

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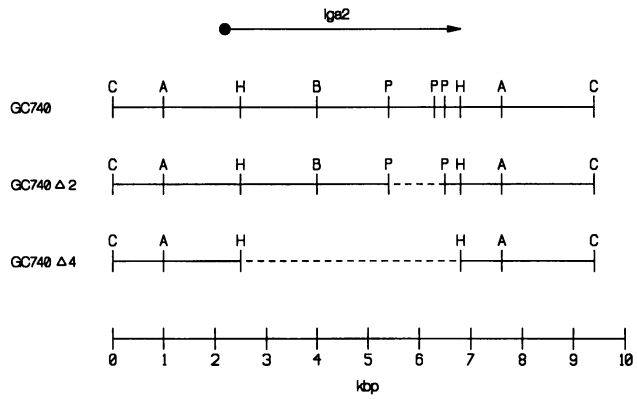

FIG. 1. Restriction maps of *N. gonorrhoeae* GCM 740 and two isogenic gene disruption mutants. Deleted regions are represented by dashed lines; both resultant constructs were negative for IgA1 protease activity. Restriction site abbreviations: A, *Ava*I; B, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I.

protease in an in vitro assay system using both wild-type gonococcal strains and IgA1 protease-deficient variants. We also examined the effect of IgA1 protease on the isolated OM of an *Escherichia coli* laboratory strain. The data presented here demonstrate that the type 2 gonococcal IgA1 protease is capable of hydrolyzing several proteins in the OMs and CMs of *N. gonorrhoeae* and further indicate that this effect is neither strain nor species limited.

(Portions of this work were presented at the 6th International Pathogenic Neisseria Conference in Pine Mountain, Ga., in 1988 [28].)

MATERIALS AND METHODS

Bacterial strains and media. *N. gonorrhoeae* GCM 740 is a prototrophic type 2 IgA1 protease-producing strain of serovar PorIB2 that has been described previously (23). A pair of Δ*iga*2 deletion-disruption mutants, GCM 740Δ4 and GCM 740Δ2, were constructed in this laboratory for use in other experiments, and their construction will be described below (Fig. 1). In GCM 740Δ2, the deletion encompasses 1.1 kbp (24% of the *iga* gene), and in GCM 740Δ4, the deletion encompasses 4.3 kbp (93% of the *iga* gene). Gonococcal strain F62 and its Iga2$^-$ isogen, F62-VD111 (PorIB7, proline requiring) have been described previously by Koomey et al. (15). As well as differing in Por serovar and auxotype, these two sets of strains have different *iga*2 genotypes, as defined by restriction site polymorphism (27). *E. coli* DH1 and HB101 were used in cloning the *iga*2 locus from GCM 740 and in construction of the deletion-disruption mutants. *Actinobacillus pleuropneumoniae* ATCC 27088 is a serotype 1-type strain.

Gonococci were propagated on GC base solid medium (Difco Laboratories, Detroit, Mich.) plus Kellogg's defined supplement (43; GCB) and grown in the defined broth medium of Morse and Bartenstein (20). The Δ*iga* mutants were isolated after transformation on GCB containing a diffusion gradient of penicillin G. Following isolation and confirmation of phenotype, these mutants were propagated on GCB. *A. pleuropneumoniae* was propagated on brain heart infusion agar and in brain heart infusion broth, both containing 10 μg of NAD (Difco) per ml. *E. coli* strains were propagated in Luria-Bertani (LB) broth or on LB agar solid medium (18) containing either ampicillin (100 μg/ml) or tetracycline (12.5 μg/ml) as necessary. Antibiotics and NAD were from Sigma Chemical Co., St. Louis, Mo.

Construction of Δ*iga* mutants. Cloning of the *iga*2 gene from GCM 740 has been reported previously (23). Standard recombinant DNA methodology (18) involving endonuclease restriction and fragment elution from agarose gels was used to construct deletion mutants (Fig. 1). A 2.4-kbp β-lactamase gene cassette was removed from pLES2, an *N. gonorrhoeae-E. coli* shuttle vector (40), and ligated into these deleted constructs in order to provide a marker which could be used to select for gonococcal transformants. Resultant Iga$^-$ Amp^r plasmids were identified and amplified in *E. coli*. Insert DNA was removed by endonuclease digestion and gel purified prior to transformation of GCM 740.

Transformation of GCM 740 was performed as previously described (1) with the following modifications. Following the transformation period (60 min at 37°C), cells were plated on GCB for a 6-h expression period at 37°C under 5% CO_2. Following the expression period, 100 μl of a penicillin G solution (20 μg/ml) was spread over the inner two-thirds of the plate and allowed to form a diffusion gradient. Penicillin-resistant colonies were isolated at 72 h and subcultured on GCB plus 0.025 μg of penicillin G per ml to verify phenotypic stability. Penicillin-resistant colonies were assayed for IgA1 protease phenotype by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{125}I-labeled IgA1 cleavage products (24). Southern blots of endonuclease-restricted genomic DNA also were probed with the 4.3-kbp *Hin*dIII *iga* fragment and the 2.4-kbp β-lactamase cassette fragment to verify the genotype of the mutants (18, 27).

Preparation of CMs and OMs. Bacteria were grown to mid-exponential phase in appropriate broth media and harvested by low-speed centrifugation. Spheroplasts were prepared by the sucrose-lysozyme-EDTA method (29) and were disrupted by ultrasonication. CM and OM fractions were separated by ultracentrifugation over isopycnic sucrose density gradients (10). The protein concentrations in each fraction were determined by using the Bio-Rad protein assay kit with bovine serum albumin as a standard (2; Bio-Rad Laboratories, Richmond, Calif.).

IgA1 protease purification and quantitation. IgA1 protease was purified from GCM 740 as previously described (39). Briefly, cell-free supernatant from cells grown in defined medium was harvested and concentrated by ultrafiltration in a positive-pressure filtration cell (Amicon Corp., Lexington, Mass.). Bulk protein was removed by batch anion-exchange treatment with DE52 at pH 7.5 (Whatman, Inc., Clifton, N.J.). Two fast protein liquid chromatography columns were used to prepare the pure IgA1 protease, a Mono-P chromatofocusing column and then a Superose 12 gel filtration column (both from Pharmacia, Inc., Piscataway, N.J.). After both column runs, fractions containing IgA1 protease activity and silver-stainable IgA1 protease were pooled, concentrated, and dialyzed into Iga2 buffer (50 mM Tris HCl [pH 7.5], 5 mM EDTA) by ultrafiltration in a Centricon-30 unit (Amicon). Determination of protease units, defined as micrograms of IgA1 cleaved minute^{-1} milliliter^{-1}, was done by quantitative SDS-PAGE analysis as described previously (32).

IgA1 protease treatment of membrane preparations. Three micrograms of protein from each membrane preparation was mixed with 1/10 volume of 10× IgA1 protease assay buffer (0.5 M Tris HCl [pH 7.5], 0.1 M $MgCl_2$, 0.1 M $CaCl_2$) and 0.17 to 4.0 U of IgA1 protease per μg of membrane protein.

In control reactions, either Iga2 buffer or heat-inactivated IgA1 protease was added instead of active enzyme. Incubation was at 37°C overnight, and the reaction was terminated by the addition of one-half volume of 2× sample buffer (16) with boiling for 5 min. Reaction mixtures were separated by discontinuous-system SDS-PAGE (16) over a 7.5 to 12.5% linear polyacrylamide gradient, followed by dual Coomassie blue-silver staining (7). Wet gels were analyzed with transillumination to detect the presence of bands which had changed in staining intensity. A decrease in staining intensity was assumed to be due to proteolysis and a resultant decrease in the concentration of protein at that position, since the buffer control lane had been treated in an identical fashion except for the presence of the protease. An increase in the staining intensity of a protein band was assumed to be due to an increase in the concentration of protein at that position.

For control experiments utilizing heat-inactivated IgA1 protease, the enzyme was inactivated by heating at 95°C for 10 min. A nonheated sample with an equivalent volume of the same preparation was used as the active-enzyme control.

Antiserum preparation and immunoblot analysis. A polyclonal antiserum was prepared in New Zealand White rabbits by standard procedures. Injections were subcutaneous, and electrophoretically pure IgA1 protease (prepared as described above) in Freund's adjuvant (Sigma) was used as the antigen. Antiserum titers were determined by assaying dilutions of the antiserum for inhibition of IgA1 protease activity in an SDS-PAGE assay (32). This antiserum preparation gives 50% inhibition of IgA1 protease activity at a dilution of 1:595.

Western blot (immunoblot) analysis was used in an attempt to determine whether the low-molecular-weight bands that increased in staining intensity were due to degradation of the exogenously supplied IgA1 protease by intrinsic membrane proteases. Following SDS-PAGE as described above, proteins were electroblotted onto a nitrocellulose membrane (BA 85; Schleicher & Schuell, Inc., Keene, N.H.) by the method of Towbin et al. (42) for 60 min at a 100-V constant voltage. Filters were blocked with 5% powdered skim milk in phosphate-buffered saline (PBS)–Tween 20 (0.05%) and probed overnight with anti-IgA1 protease antiserum. Washes were in PBS-Tween 20, and ^{125}I-labeled protein A was used to detect antigen-antibody complexes, with exposure to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Human serum proteins. Human serum albumin (Cohn fraction V) and human gamma globulins (purified from Cohn fractions II and III) were purchased from Sigma. The human IgA1 and IgA2 paraproteins (Mor and Mapp, respectively) were purified from patient sera by standard procedures.

Digestion of human serum proteins. Aliquots of purified type 2 IgA1 protease from *N. gonorrhoeae* GCM 740 (0.5 U) were incubated overnight with 0.25 μg of IgA1 (Mor), 0.25 μg of IgA2 (Mapp), 0.50 μg of gamma globulins, or 0.25 μg of albumin in a reaction mix containing 50 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, and 10 mM $CaCl_2$. Reactions were terminated by the addition of one-half volume of double-strength SDS-PAGE sample buffer (16) and boiling for 5 min. Reaction products were resolved over either 9.0% or 7.5 to 12.5% SDS-PAGE gels, stained, and analyzed with transillumination as described above for any degradation which had occurred in the presence of the added IgA1 protease.

RESULTS

Specificity of purified IgA1 protease preparation. The purified IgA1 protease preparation was demonstrated to be specifically active against a human IgA1 paraprotein (Mor) and to lack proteolytic activity against (i) a human IgA2 paraprotein (Mapp), (ii) a human gamma globulin preparation, and (iii) human serum albumin following overnight incubation (data not shown).

Incubation of isolated OMs with exogenous IgA1 protease. OMs of GCM 740 and its two isogenic IgA1 protease-negative derivatives, GCM 740Δ2 and GCM 740Δ4, were incubated with electrophoretically pure type 2 gonococcal IgA1 protease prepared from GCM 740 to determine whether this enzyme could proteolyze gonococcal OM proteins. Figure 2 shows a typical silver-stained SDS-PAGE gel comparing the protein compositions of OMs incubated either with or without exogenous pure protease.

Two proteins of 78 and 68 kDa were proteolyzed, i.e., decreased in staining intensity, in all three of the variants in the GCM 740 set (Fig. 2, lanes 4, 6, and 8). In contrast, an increase in staining intensity was observed for bands of 61, 38, 24.5, and 14 kDa in all three of these variants. In addition, a band of 88 kDa which was present in the wild type (lanes 3 and 4) and missing in both mutants in the absence of exogenous protease was observed to increase in staining intensity for both of the mutant variants (lanes 6 and 8). Control experiments in which OMs were incubated with either buffer alone (Fig. 2, lanes 3, 5, and 7) or with heat-inactivated IgA1 protease (data not shown) indicated that the proteolytic activity observed was heat labile and associated with the addition of electrophoretically pure IgA1 protease. Immunoblot analysis with anti-IgA1 protease serum demonstrated that the lower-molecular-weight bands which appeared post-IgA1 protease treatment were not degradation products of the added IgA1 protease (data not shown).

On the basis of relative staining intensity, all of the proteins which were affected by the addition of protease were minor components of the OM. The identities of these protein bands are not known. However, it was determined by SDS-PAGE analysis and with heat and reduction modification experiments that they did not correspond to some of the previously described gonococcal OM proteins or antigens; i.e., they were not Por (PI), Rmp (PIII), or Opas (PIIs) on the basis of apparent molecular weights (9, 41).

To determine whether proteolysis of gonococcal OM proteins was strain specific, outer membranes of *N. gonorrhoeae* F62, a type 2a IgA1 protease producer (27), and its isogenic protease-negative mutant, F62-VD111, were incubated with purified type 2b protease from GCM 740.

When the F62 set was analyzed, the two variants had in common a single proteolyzed band of 48 kDa (Fig. 2, lanes 10 and 12). Protein bands of 24.5 and 14 kDa increased in staining intensity for both of these variants, and these corresponded to bands of the same molecular mass also observed to increase in the GCM 740 set. The IgA1 protease-deficient mutant, F62-VD111, exhibited a band of 58 kDa which increased in staining intensity following treatment with protease (Fig. 2, lane 12). As stated above, the identities of these bands were not determined, but some identities were ruled out based on apparent molecular weight and behavior.

Incubation of isolated CMs with exogenous IgA1 protease. Experiments with CMs were performed in a fashion similar to those with the OMs to determine whether the observed

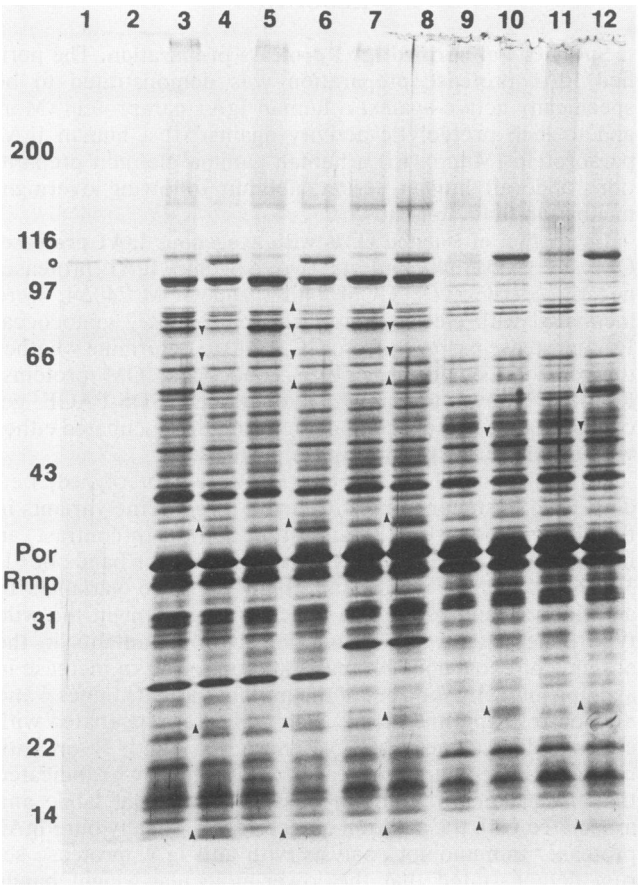

FIG. 2. Silver-stained SDS-PAGE gel of *N. gonorrhoeae* OMs following incubation with and without exogenous pure gonococcal type 2 IgA1 protease. OM proteins (3 μg) were incubated for 14 h at 37°C with either Iga2 buffer or 12 protease units of IgA1 protease in Iga2 buffer. Following incubation, mixtures were boiled for 5 min in sample buffer and separated over a linear 7.5 to 12.5% gradient SDS-PAGE gel. Lanes: 1, 12 U of IgA1 protease; 2, 12 U of IgA1 protease after 14 h of incubation at 37°C; 3 and 4, GCM 740; 5 and 6, GCM 740Δ4; 7 and 8, GCM 740Δ2; 9 and 10, F62; 11 and 12, F62-VD111. Odd-numbered lanes contain OMs incubated with Iga2 buffer, and even-numbered lanes contain OMs incubated with protease. Downward-pointing arrowheads indicate bands which decreased in intensity following incubation with protease and upward-pointing arrowheads indicate bands which increased in intensity. Positions of molecular mass standards (Bio-Rad) are shown along the left in kilodaltons, as are those of the major gonococcal OM proteins (Por and Rmp) and IgA1 protease (o).

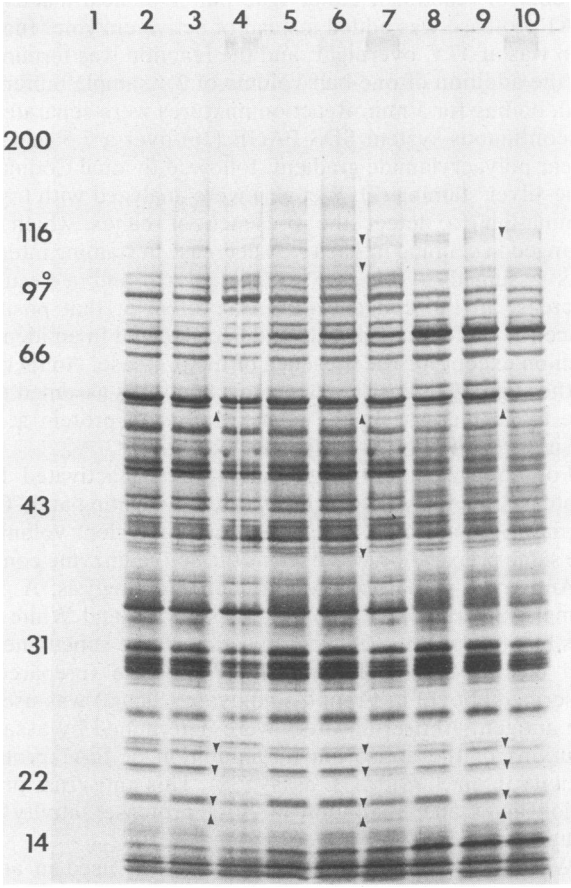

FIG. 3. Silver-stained SDS-PAGE gel of *N. gonorrhoeae* CMs following incubation with Iga2 buffer, heat-inactivated type 2 gonococcal IgA1 protease in Iga2 buffer, and active IgA1 protease in Iga2 buffer. CM proteins (3 μg) were incubated for 13.5 h at 37°C as described below. Following incubation, mixtures were boiled for 5 min in sample buffer and separated over a linear 7.5 to 12.5% gradient SDS-PAGE gel. Lanes: 1, 6 U of IgA1 protease after 13.5 h of incubation at 37°C; 2, 3, and 4, GCM 740; 5, 6, and 7, GCM 740Δ4; 8, 9, and 10, GCM 740Δ2. Lanes 2, 5, and 8 contain CMs plus buffer; lanes 3, 6, and 9 contain CMs plus inactivated protease (6 protease unit equivalents); and lanes 4, 7, and 10 contain CMs plus 6 U of protease (2 U per microgram of CM protein). Downward-pointing arrowheads indicate bands which decreased in intensity following incubation with protease, and upward-pointing arrowheads indicate bands which increased in intensity. Positions of molecular mass standards (Bio-Rad) are shown along the left in kilodaltons, as is that of IgA1 protease (o).

proteolysis was limited to OM proteins. We found that there were also CM proteins which changed in staining intensity following incubation with exogenous, pure IgA1 protease (Fig. 3, lanes 4, 7, and 10). Control incubations with buffer alone or with heat-inactivated IgA1 protease demonstrated that this proteolysis was heat labile and dependent upon the addition of active IgA1 protease (Fig. 3, lanes 3, 6, and 9).

When the GCM 740 isogenic set was analyzed (Fig. 3, lanes 2 to 10), three protein bands of 24.5, 23.5, and 21.5 kDa were proteolyzed and two protein bands of 57 and 20.5 kDa were observed to increase in intensity in all three variants. The two mutants, GCM 740Δ4 and GCM 740Δ2, also shared a protein band of 121 kDa which was proteolyzed (lanes 7 and 10). Strain GCM 740Δ4 exhibited two protein bands of 107 and 39 kDa which were proteolyzed (lane 7); these protein bands were not observed for either GCM 740 or GCM 740Δ2.

When analyzed in a similar experiment, the F62 set of variants showed a single CM protein band of 45 kDa in both the wild type and the mutant which increased in staining intensity following protease treatment (data not shown).

Incubation of *E. coli* OMs with exogenous IgA1 protease. To determine whether the observed proteolysis was restricted to gonococcal OM proteins, OMs of two other gram-negative bacteria, *E. coli* DH1 and *A. pleuropneumoniae*, were incubated with purified type 2 gonococcal IgA1 protease. Several *E. coli* DH1 OM proteins changed in staining intensity following incubation with the protease (Fig. 4). *E. coli*

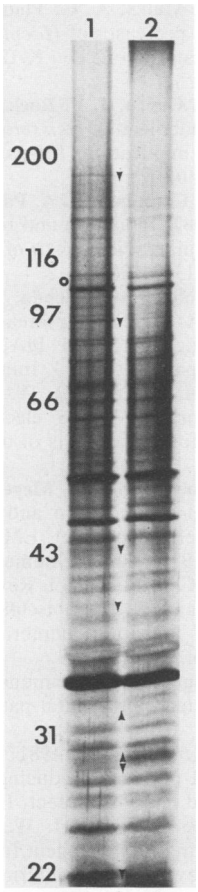

FIG. 4. Silver-stained SDS-PAGE gel of *E. coli* DH1 OMs following incubation with and without exogenous pure type 2 gonococcal IgA1 protease. OM proteins (3 μg) were incubated for 14 h at 37°C with either Iga2 buffer or IgA1 protease (0.17 protease unit per microgram of OMs) in Iga2 buffer. Following incubation, mixtures were boiled for 5 min in sample buffer and separated over a linear 7.5 to 12.5% gradient SDS-PAGE gel. Lane 1, OMs plus Iga2 buffer; lane 2, OMs plus protease. Downward-pointing arrowheads indicate bands which decreased in intensity following incubation with protease, and upward-pointing arrowheads indicate bands which increased in intensity. Positions of molecular mass standards (Bio-Rad) are shown along the left in kilodaltons, as is that of IgA1 protease (o).

DH1 OM bands which were proteolyzed included protein bands of 177, 90, 43, 38.5, 27.5, 22.5, and 19.7 kDa. Two minor bands of 31 and 28.5 kDa increased in staining intensity. *A. pleuropneumoniae* OMs exhibited a band of 37 kDa which was cleaved and two bands of 33 and 25 kDa that increased in staining intensity (data not shown).

While the identities of these protein bands are unknown, the fact that some protein bands are observed to be degraded while others increase in staining intensity supports the hypothesis that the type 2 gonococcal IgA1 protease has the ability to cleave OM proteins of gram-negative species other than *N. gonorrhoeae*.

DISCUSSION

In this study, we demonstrated that the type 2 IgA1 protease of *N. gonorrhoeae* is capable of cleaving proteins found in both the OMs and CMs of the gonococcus and also of cleaving OM proteins of other gram-negative bacteria. This proteolytic activity was associated with a silver-stainable, electrophoretically pure, heat-labile preparation of IgA1 protease. Proteolysis of bacterial membrane proteins or of any substrate of bacterial origin other than the protease itself by any IgA1 protease has not been reported previously.

Our conclusion that the phenomenon observed, i.e., the disappearance of some proteins and appearance of others in membrane preparations incubated with IgA1 protease, is due to specific proteolysis of membrane proteins by this enzyme is supported by the results of several control experiments. First, this phenomenon requires addition of active IgA1 protease; incubation of OM or CM preparations with heat-inactivated protease led to no alteration of proteins. Second, the appearance of new protein bands was not due to autodegradation of the IgA1 protease, as evidenced by the integrity of the pure protease after incubation overnight at 37°C (Fig. 2, lanes 1 and 2). Third, the appearance of new proteins was not due to degradation of the added protease by any hydrolytic activity present in the membrane preparations; immunoblot experiments demonstrated no bands other than the 109-kDa intact protease reactive with monospecific antiserum against IgA1 protease. Fourth, the lack of nonspecific, contaminating proteolytic activity in the purified IgA1 protease preparation was demonstrated by the lack of digestion of some relevant human serum proteins, including human IgA2.

We constructed Iga^- isogenic variants specifically to allow us to examine membrane proteins produced in the absence of protease as potential substrates. The ability to isolate and propagate these IgA1 protease-deficient *N. gonorrhoeae* variants indicates that neither the ability to produce IgA1 protease nor the events of enzyme production and secretion are required for in vitro growth of the gonococcus. Our Iga^- mutant variants grow equivalently to the wild type in standard broth media and are capable of growing anaerobically and under iron limitation and of expressing the appropriate previously defined stress response OM proteins (4, 5, 37, 38). However, we did observe some protein profile differences between the wild type and Iga^- isogenic variants (Fig. 2, lanes 3, 5, and 7) (28, 38). We initially hypothesized that these differences would be "cured" by the addition of protease to the membrane preparations. This did occur in the case of an 88-kDa OM protein present in wild-type GCM 740 but not in the mutants in the absence of protease and found in both mutants after incubation with active protease. However, in general the proteins which differed between the wild type and mutant variants were not apparent substrates for the protease.

Most of the altered proteins, whether in the OM or CM, were found in all members of an isogenic set. This is strongly supportive of specific proteolysis of unique common substrate proteins rather than generalized degradation. The presence of IgA1 protease-susceptible proteins in the OM and CM of wild-type gonococcal strains (including both GCM 740 and F62) which produced active enzyme, as well as in the *iga* mutants, may be due to the in vitro accessibility of both sides of the OM and CM. In contrast, in an in vivo situation the active form of IgA1 protease, as measured against human IgA1, is restricted to the extracellular fluids (33). However, during the process of secretion, the intact IgA1 protease proenzyme (enzyme plus α and β peptides) does exist in the periplasmic space and therefore has access to the periplasmic sides of both the OM and CM. While it has been reported that this intermediate form of the enzyme has no activity against human IgA1, it is not known whether the

proenzyme has proteolytic activity against any other substrate (33). It is therefore possible that proteolysis of membrane proteins not exposed on the cell surface could occur in vivo.

We do not know the specific identities of any of these membrane proteins that are degraded by IgA1 protease. However, we do know that none of them are among the previously well-described gonococcal major OM proteins, i.e., Por, Rmp, and Opa. We also have examined the susceptibility of defined iron-limitation- and anaerobiosis-induced OM proteins and have found that these stress-induced proteins also are not substrates for IgA1 protease hydrolysis (38). The data suggest that some of the substrate proteins may be conserved gonococcal membrane proteins, since degradation products of the same apparent molecular weights were observed in all five variants examined (Fig. 2).

Alteration of protein bands in both the GCM 740 and F62 sets of strains by protease purified from GCM 740 indicated that the proteolysis of membrane proteins is not strain specific (Fig. 2). This proteolytic effect is also not restricted by species, as demonstrated in Fig. 4, which shows that OM proteins of *E. coli* DH1 are susceptible to cleavage by exogenously added IgA1 protease. Similar results were observed when isolated OMs of a porcine pathogen, *A. pleuropneumoniae*, were treated with IgA1 protease.

The fact that there are proteins in the OM of *N. gonorrhoeae* that are permissive substrates for the IgA1 protease prompts the question of whether the enzyme performs this function in vivo. If membrane proteins were processed in vivo by IgA1 protease, one would expect that either there would be proteins found in the mutants (and not in the wild type) which would be degraded by added protease or that there would be proteins absent in the mutants (and found in the wild type) that would appear after incubation with added protease. The presence of the 88-kDa OM protein in wild-type GCM 740 but not in the mutants, and its appearance after IgA1 protease treatment of the mutants, suggests that this type of processing of membrane proteins by IgA1 protease does occur in vivo.

In summary, the data presented here indicate that there are proteins present in the OMs and CMs of *N. gonorrhoeae* that are permissive in vitro substrates for gonococcal type 2 IgA1 protease. Proteolysis of these membrane proteins was dependent on the addition of active IgA1 protease and was neither strain nor species restricted. This report defines an entire range of new potential substrates for the IgA1 proteases, whose role in the physiology of the organism or the pathogenesis of gonococcal infection remains to be determined.

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