IgA protease of *Neisseria gonorrhoeae*: isolation and characterization of the gene and its extracellular product

Roman Halter, Johannes Pohlner and Thomas F. Meyer

Max-Planck-Institut für Medizinische Forschung, Abteilung Molekulare Biologie, and Zentrum für Molekulare Biologie der Universität, Jahnstrasse 29, 69 Heidelberg, FRG

Communicated by U. Henning

Gonococcal virulence is thought to rely on multiple characteristics including the production of an extracellular protease specific for human IgA1. Using a sensitive filter assay we have isolated an *Escherichia coli* clone which harbours the gene of *Neisseria gonorrhoeae* MS11 IgA protease on a multi-copy number plasmid. This clone secretes IgA protease activity to an extent similar to that of the parental MS11 strain. By exonucleolytic digestion of the cloned insert we obtained a fragment of 4.6 kb which could not be shortened further without loss of IgA protease expression. Compared with the cloned IgA protease gene from *N. gonorrhoeae* F62, this minimal gene segment shows marked differences in the arrangement of restriction sites. We suppose that these differences determine strain-specific variations of *N. gonorrhoeae* IgA proteases and also affect the secretory properties of the enzyme when produced in *E. coli*. A novel purification procedure developed for IgA protease of *N. gonorrhoeae* allowed us to correlate the enzyme activity with a distinct protein band in SDS acrylamide gels. By comparison with the enzyme prepared from the *E. coli* clone, we identified a 105-kD protein as the extracellular form of gonococcal IgA protease. Key words: *Neisseria gonorrhoeae*/IgA protease/gene/restriction site polymorphism/secretion

Introduction

A number of medically important bacteria which invade the human mucosa release an extracellular protease which specifically cleaves human IgA1 (for review, see Kornfeld and Plaut, 1981). These pathogens include *Neisseria gonorrhoeae*, the causative agent of gonorrhea, as well as *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, which primarily are responsible for bacterial meningitis and pneumonia infections. A variety of bacteria associated with human urinary tract infections also are frequently found to be IgA protease positive (Milazzo and Delisle, 1984). In contrast, non-pathogenic *Neisseria* and *Haemophilus* species, as well as opportunistic bacteria such as the majority of clinical isolates of *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, do not exhibit IgA protease activity.

The secretory immune system, which is spatially and functionally distinct from the immunity at non-mucosal sites, relies mainly on IgA antibody which is thought to counteract bacterial invasion by preventing adherence of bacteria to epithelial cells (Williams and Gibbons, 1972). Human IgA consists of two subclasses differing in their hinge region; a duplicated octameric amino acid sequence found in IgA1 is missing in IgA2 (Frangione and Wolfenstein-Todel, 1972;

Kratzin *et al.*, 1978). This duplication is the target for all known IgA proteases and cleavage results in the formation of intact Fab and Fc fragments (Mulks *et al.*, 1980a; Plaut *et al.*, 1977).

The role of IgA protease in bacterial virulence is still unclear. Therefore efforts have been made to approach the IgA protease problem by molecular genetics. Koomey *et al.* (1982) reported the isolation of a gonococcal IgA protease gene which was used to construct an *N. gonorrhoeae* strain deficient in IgA protease production. This cloned IgA protease gene which originated from *N. gonorrhoeae* F62 exhibits relatively little enzyme activity in *E. coli*. Similarly, an IgA protease clone derived from *H. influenzae* accumulates active enzyme in the periplasmic space of *E. coli* (Bricker *et al.*, 1983). We cloned an IgA protease gene with different characteristics from *N. gonorrhoeae* MS11 and achieved efficient expression and extracellular secretion of the enzyme in *E. coli*. A purification procedure designed for gonococcal IgA protease enabled us to identify the extracellular gene product.

Results

Cloning of the IgA protease gene

Chromosomal DNA from *N. gonorrhoeae* MS11 was digested with a combination of two restriction enzymes to produce fragments bearing non-identical ends. These fragments were size-fractionated and inserted into a pBR322 derivative which had been previously treated with the corresponding restriction enzymes. The hybrid plasmid DNA was transformed into *E. coli* GC1 and the transformed clones were directly screened for IgA protease activity by using a specific filter assay. This filter assay is based on the cleavage of radioactive IgA antibody (bound to nitrocellulose via its secretory component) and allows easy detection of IgA protease secreting clones among hundreds of transformants on a single culture plate (see Figure 1). In an experiment using gonococcal DNA restricted with *SalI* and *Clal*, we obtained one positive clone out of ~100 transformants. Plasmid DNA from this clone was transferred to *E. coli* DH1 (*recA*) and isolated from this strain in large quantities.

Structural variations among gonococcal IgA protease genes

Restriction mapping of the IgA protease encoding hybrid plasmid (pIP1) revealed an inserted *Clal/SalI* fragment of 9.3 kb. A physical map of pIP1 is given in Figure 2A. The identity of the cloned DNA segment to the corresponding chromosome segment was verified by Southern blotting (Figure 3). From the hybridization experiment also a physical map of the corresponding genomic region was deduced (Figure 2A).

It is an intriguing observation that the restriction map of the IgA protease gene isolated from *N. gonorrhoeae* F62 by Koomey *et al.* (1982) differs significantly from our clone regarding the distribution of restriction sites although their overall arrangement appears to be reminiscent. Besides single

*Cla*I and *Pst*I sites, two *Hind*III sites surrounding a 4.2-kb fragment are common to both genes, whereas several other restriction sites, e.g. *Bgl*II and *Hpa*I cleavage sites, differ in their abundance and relative location. We suppose that this restriction site polymorphism reflects even more extensive divergences at the nucleotide level of the two genes. Since the variations are predominantly found in a region essential for

IgA protease expression (Figure 2A), they may significantly influence the features of the enzyme.

Our hybridisation data (and those of Koomey and Falkow, 1984) clearly show that, using cloned IgA protease genes as probes for chromosomal hybridisations, only a single chromosomal gene locus can be revealed in *N. gonorrhoeae* MS11 (Figure 3). Thus, the IgA protease genes isolated from *N. gonorrhoeae* MS11 and F62 must be unique genes that discriminate from each other by strain-specific characteristics. At this point we do not exclude the possibility that gonococcal IgA protease is subject to frequent variations as it is observed for the pilus and opacity proteins of this bacterium (Salit *et al.*, 1980). The mechanism by which this might occur should be different for these surface proteins since, in contrast to the IgA protease gene, their genes possess numerous related gene sequences in the chromosome which are thought to be involved in the production of variant expression copies (Meyer *et al.*, 1982; Stern *et al.*, 1984).

Gene sequences essential for IgA protease expression

To define the gene sequences in our clone which are required for synthesis and secretion of active IgA protease by *E. coli*, the cloned insert was exonucleolytically digested with *Bal*31 enzyme from both directions in two separate experiments and recloned in the same vector. A series of transformed colonies harbouring truncated IgA protease inserts were tested for enzyme expression using the filter assay. Subsequent restriction analysis of expression positive and negative plasmids allowed us to determine the limits of essential IgA protease gene sequences within 100 bp at both ends (Figure 2A). The minimum sequence information required for enzyme expression and secretion turned out to be 4.6 kb, a segment which possesses a potential coding capacity of ~1500 amino acids or 165 kd of protein.

Two of the smallest plasmid clones which still allowed normal IgA protease expression and which were truncated from one of the ends were used to construct a minimal gene by cleavage and rejoining at the unique *Bgl*II site which is located in the middle of the coding area. The resulting clone,

Fig. 1. Filter assay for IgA protease. (A) shows bacterial colonies of *E. coli* DH1 harbouring *Bal*31-truncated pIP1 DNAs some of which are deficient in IgA protease expression. (B) represents an autoradiograph of an IgA1-coated nitrocellulose filter which was exposed to the colonies shown in part A. Solid arrows indicate a few IgA protease positive clones, the dotted arrow points to a negative *E. coli* clone.

Fig. 2. Physical map of gonococcal IgA protease genes. (A) IgA protease gene of *N. gonorrhoeae* MS11. The double line section shows the 9.3-kb *Cla*I/*Sal*I fragment as cloned in plasmid pIP1. Sequences which are essential for IgA protease expression are indicated by a solid line. This region is contained in subclone pIP100 and results from exonucleolytic digestion of pIP1 at both ends. (+) and (-) concerns IgA protease expression of subclones which are truncated at the indicated position. Vector DNA is omitted in this drawing. Instead, the map of pIP1 is extended at the ends by restriction data obtained from chromosomal Southern blot analysis (thin line). (B) IgA protease gene of *N. gonorrhoeae* F62 (adapted from Koomey *et al.*, 1982). The restriction map is aligned to part (A) of the figure in a way such that a maximum of apparent coincidence in the restriction sites is obtained (compare *Cla*I, *Hind*III and *Pst*I sites). IgA protease expression in the F62 gene is reported to take place in a region covering the two *Hind*III sites (Koomey and Falkow, 1984).

Fig. 3. Genomic Southern hybridizations with the IgA protease gene as probe. Chromosomal DNA of *N. gonorrhoeae* MS11 was digested with various restriction enzyme combinations and hybridized with pIP1 as a probe. Single lanes represent chromosomal digests with *Clal* only (a) or *Clal* in addition to *EcoRI* (b), *HindIII* (c), *PstI* (d), *Aval* (e), *SmaI* (f), *XhoI* (g), *BclI* (h), *HpaI* (i) and *PvuII* (k). The size markers (kb) are phage λ *HindIII* fragments.

named pIP100, was still active (Figure 2A).

To determine the number of independent gene functions encoded by this relatively large minimal segment, we performed a complementation experiment by introducing two defective plasmids into the *recA*⁻ host DH1. One of the plasmids with an insert missing ~80 bp at its left end carried a tetracycline resistance gene, the other lacked 100 bp of essential sequence information at its right end and carried an ampicillin resistance gene. These two plasmids, both carrying the pBR origin, were stably maintained in the same *recA* bacterium by selection on ampicillin and tetracycline but no IgA protease activity could be detected. In contrast, the control experiment using a *recA*⁺ host revealed some recombinants which were IgA protease positive. Furthermore, to exclude sequence aberrations within the two defective plasmid clones, their end segments were reconstituted by inserting small *EcoRI*/*HindIII* or *PstI*/*BamHI* border fragments of pIP100. Both reconstituents expressed the enzyme. Although this experiment excludes the existence of a second independent, *trans*-complementable gene function, it does not rule out the linked expression of two gene products via a polycistronic IgA protease operon.

Expression and secretion of IgA protease by the *E. coli* clone
Gonococcal IgA protease activity was determined by a semi-quantitative assay which used radioactively-labelled secretory IgA from human colostrum as a substrate. After treatment with enzyme, the reaction products were analysed by gel electrophoresis. Since the enzyme selectively cleaves IgA1, the fraction of IgA2 contained in the crude preparation of secretory IgA cannot be converted into fragments (see Figure 4A as an example). Human IgG and IgM are also left intact by the enzyme. According to these criteria, the enzymes from culture supernatants of *N. gonorrhoeae* MS11 and *E. coli* GC1 (pIP1) are identical. Since IgA proteases are believed to

Table I. Secretion of MS11 IgA protease by *E. coli*

Percentage of enzymatic activity

extracellular environment	periplasmic space	cell lysate
97	1	2

3 ml of a late-logarithmic-phase GC1 (pIP1) culture were harvested. The supernatant was saved as extracellular fraction. The cells were washed once in growth medium and then either suspended in 0.5 ml growth medium and disintegrated by sonication (cell lysate) or subjected to osmotic shock (periplasmic fraction) and adjusted to the same volume.

Table II. Isolation of IgA protease from *N. gonorrhoeae* growth medium

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Fraction I					
growth medium	10 000	35	4000	114	100
Fraction II					
ammonium sulfate precipitate	8.5	34	3400	100	85
Fraction III					
gel filtrate	41	8.6	3300	383	84
Fraction IV					
BioRex 70 eluate	4.5	0.3	675	2250	17

Purification procedure: *N. gonorrhoeae* MS11 (P⁻, O⁻) was cultivated in 10 l of Catlin's defined medium by gentle shaking at 37°C. At an OD 590 of 0.4 the bacteria were removed by centrifugation. The supernatant (Fraction I) was cooled to 4°C, supplemented with 50 µg/ml chicken ovalbumin, and the protein precipitated by the addition of 0.6 g ammonium sulfate/ml solution. After 8 h at 4°C the precipitate was collected by centrifugation at 20 000 g and dissolved in 35 ml buffer A (300 mM NaCl, 10% glycerol, and 20 mM Tris-Cl, pH 8.1). The solution was clarified by centrifugation and the protein precipitated once more by addition of 0.6 g ammonium sulfate to each ml. The precipitate was collected, redissolved in 4 ml buffer A, and dialyzed against the same buffer overnight. This fraction (Fraction II) was applied to an Ultrogel AcA44 (LKB) column (2.6 cm in diameter, 120 cm in length) equilibrated in buffer A and eluted with the same buffer. Fractions of 4.2 ml were collected and enzyme activity was usually recovered between fraction 42 and 54 (see Figure 4A). Active fractions were pooled (Fraction III), and the protein was concentrated by precipitation with ammonium sulfate under the same conditions as described above. The precipitate was dissolved in 4 ml buffer B (50 mM K-phosphate, pH 6.5, and 10% glycerol) and dialyzed against this buffer overnight. The enzyme was then applied to a 0.4 ml BioRex 70 (BioRad) column (0.6 cm in diameter and equilibrated in buffer B). The column was washed with 2 ml buffer B and developed by a linear phosphate gradient of 50–300 mM K-phosphate in buffer B (2 × 5 ml). Fractions of 0.5 ml were collected and assayed for IgA protease activity. The enzyme eluted between 80 and 220 mM K-phosphate (Figure 4B). Active fractions were pooled, dialysed against buffer C (20 mM K-phosphate, pH 7, 50% glycerol), and stored at -20°C (Fraction IV). One unit was defined as the amount of enzyme required to achieve 50% cleavage of labelled IgA in the standard assay within a period of 15 min.

be metallo-enzymes with a variable sensitivity to EDTA (Plaut *et al.*, 1975, 1977) we tested this parameter as well. For both enzyme fractions, a slight decrease in enzyme activity is observed at EDTA concentrations over 50 mM.

The high enzymatic activity found in culture supernatants of *E. coli* GC1 (pIP1) is a surprising result. Quantitative measurements showed approximately equivalent secretion of the *E. coli* clone and *N. gonorrhoeae* MS11. To analyze whether all the synthesized IgA protease in *E. coli* is transported through the inner and outer membranes, we measured the amount of enzyme activity in cytoplasm and periplasm in

Fig. 4. IgA protease elution profiles. **(A)** gel filtration through Ultrogel AcA44: the conditions for enzyme purification are described in Table II. Open circles indicate the protein concentration determined according to Bradford (1976). Closed circles indicate IgA protease activity in % as evaluated by cleavage of labelled IgA (see Materials and methods). The results of this assay are shown at the bottom of section A. 10 μ l of each fraction were incubated with labelled IgA for 2 h. Production of IgA1 Fab α 1 fragment can be detected in fractions 42 to 54. Further radioactive bands represent secretory component (sc), IgA heavy chain (h), and IgA light chain (l). **(B)** BioRex70 chromatography: (details are presented in Table II). Protein concentration was evaluated by the silver stain method of Switzer *et al.* (1979) in the acrylamide gel (open circles). (+) indicates the concentration of K-phosphate in the effluents as measured by conductivity. To determine IgA protease activity 1 μ l of each fraction was incubated with labelled IgA for a period of 20 min (closed circles). Fraction a and b represent flow through and wash with a total volume of 4 ml and 2 ml, respectively.

comparison with the extracellular environment. The results (shown in Table I) indicate almost complete secretion of the enzyme by *E. coli* as in the case of *N. gonorrhoeae*.

Hybrid *E. coli* cells do not seem to produce large quantities of IgA protease in the stationary phase, since only small amounts of activity can be detected after prolonged incubation of cells in fresh medium. Consistently, we were not able to detect IgA protease activity or a radioactively-labelled protein band which could be definitely connected with the enzyme in supernatants of *E. coli* DS410 (pIP1) 35 S-labelled minicells, and we therefore decided to identify the IgA protease by means of enzyme purification from viable cells.

Purification of IgA protease from culture supernatants

Although several reports deal with the preparation of IgA proteases from various pathogenic species no purification procedure leading to a definitive identification of the extracellular enzyme was available. It does appear though, that IgA proteases are large metallo-enzymes which are secreted by the microorganisms in small quantities of high enzymatic activity. In contrast to IgA proteases from other organisms, the *Neisseria* enzymes are characterised by their basic isoelectric point (Plaut *et al.*, 1978). We made use of this property of the gonococcal enzyme in a cation-exchange step which is an essential part of the following purification procedure.

To avoid contamination of the extracellular enzyme by constituents of the culture medium, we used a chemically defined medium for growth of a non-piliated MS11 variant. The supernatant of a culture in its late logarithmic phase was supplemented with chicken ovalbumin to facilitate precipitation of the IgA protease with ammonium sulfate. The precipitate was dissolved in buffer and applied to an Ultrogel column for gel filtration. In a third step, active fractions from this column were combined, dialysed against low salt buffer, and loaded on a small BioRex 70 column. The enzyme was eluted by a linear salt gradient. A detailed description is given in Table II and the elution profiles of the enzyme are shown in Figure 4.

Identification of extracellular IgA protease

The effluents and the combined active fractions of each of the purification steps were tested for their ability to cleave radioactive secretory IgA1 and for their protein composition on SDS polyacrylamide gels. A distinct double band of ~105 and 106 kD strictly correlated with the enzymatic activity of IgA protease. The native mol. wt. of MS11 IgA protease was determined by sedimentation through a glycerol gradient (Figure 5). This method revealed a size of ~105 kD for the enzyme. Corresponding results were obtained by filtration of purified enzyme through a small Ultrogel AcA 44 column (as

Fig. 5. Sedimentation of IgA protease through glycerol. Sedimentation was performed as outlined in Materials and methods. The sedimentation profile of Fraction IV MS11 IgA protease (closed circles) represents the enzymatic activity as well as the appearance of a 105/106-kd double band in the SDS acrylamide gel. The mol. wts. (kd) on top indicate the position of the internal marker proteins after sedimentation. The straight line shows the evaluation of the mol. wt. for IgA protease on a logarithmic scale (open circles).

described in Materials and methods).

To obtain final proof of the identity of the enzyme, we isolated gonococcal IgA protease from *E. coli* GC1 (pIP1) grown in casamino medium by using the same purification procedure as for the gonococcal strain. The purified protein shows the characteristics of gonococcal IgA protease. It was subjected to gel electrophoresis and compared with the enzyme fraction from MS11 (Figure 6). In contrast to the MS11 enzyme, which shows a double band at 105/106 kd, the cloned protease migrates as a single 105-kd band. Since there is no other visible protein band co-migrating in both preparations we conclude that the smaller protein band of 105 kd represents MS11 IgA protease. The molecular significance of the slightly larger protein in the gonococcal preparation is as yet not known.

Discussion

This report describes the isolation of the gene coding for IgA protease of *N. gonorrhoeae* MS11 and the identification of the extracellular enzyme. The screening procedure for an IgA protease secreting *E. coli* clone was much facilitated by using a novel filter assay. Comparison of the IgA protease preparations from *N. gonorrhoeae* MS11 and an *E. coli* strain containing the cloned gene revealed a single common protein co-purifying with the IgA protease activity. The assigned mol. wt. of 105 kd for the denatured as well as the native conformation of the extracellular protein appears to contradict the value of 140 kd reported for the *N. gonorrhoeae* F62 IgA protease, which was obtained by labelling intracellular protein of *E. coli* minicells (Kooimey *et al.*, 1982). At least one other secretory protein of a Gram-negative bacterium, the *E. coli* hemolysin (Goebel and Hedgpeth, 1982), has been shown to be drastically truncated during its secretion. We believe that the IgA protease is also processed during its transport through the inner and outer membranes and that this accounts for the difference in its intra- and extra-cellular mol. wt. This view is consistent with our finding that the minimal

Fig. 6. Comparison of purified IgA protease preparations from MS11 and GC1 (pIP1). Approximately 0.4 μ g of IgA protease purified from the gonococcal strain (a) and the *E. coli* hybrid (b) were loaded on a 10% acrylamide gel. Staining was performed by the method of Switzer *et al.* (1979). Mol. wt. markers (BioRad) are indicated at the left side (kd). The IgA protease preparations exhibit a single common band of a mol. wt. of 105 kd.

gene segment required for IgA protease expression harbours an extra coding capacity for ~60 kd of protein which is not recovered in the extracellular form of IgA protease. The final verification of this hypothesis depends on the isolation of the putative pre-protease from the bacterial cytoplasm and also on a detailed analysis of the IgA protease gene.

The observation that *N. gonorrhoeae* MS11 IgA protease is almost completely secreted by *E. coli* promises to open up a valuable model system for the investigation of extracellular secretion of proteins by a procaryotic cell. Although a few naturally occurring *E. coli* isolates are known to produce extracellular IgA protease (Milazzo and Delisle, 1984), previous experiments have shown that IgA protease encoding gene segments derived from a number of other Gram-negative bacterial species and cloned in *E. coli* secrete the enzyme insufficiently (Kooimey *et al.*, 1982; Bricker *et al.*, 1983; J. Pohlner, unpublished data). A detailed comparison of these genes might therefore help to define the characteristics required for extracellular secretion in *E. coli*.

The difference in the arrangement of restriction sites in the IgA protease genes of *N. gonorrhoeae* MS11 and strain F62 (Kooimey *et al.*, 1982), obviously indicates a strain-specific variation. Our Southern blotting experiments show a single corresponding chromosomal fragment in *N. gonorrhoeae* MS11 when the IgA protease plasmid pIP1 was used as a

probe. Since the clone of Koomey and co-workers exhibits at least a few common characteristics with pIP1 we do not believe that the cloned inserts are derived from two different chromosomal loci in *N. gonorrhoeae*. Instead, preliminary hybridization data show that the IgA protease genes of independent gonococcal isolates often differ from each other in the arrangement of restriction sites (R. Halter and J. Pohlner unpublished data). Similar results demonstrating inter-species differences in the structure of IgA protease genes were recently reported by Koomey and Falkow (1984). We suggest that such differences in the restriction pattern reflect alterations in the nucleotide sequence of the IgA protease gene and may account for the diversity in serological and/or cleavage site specificity reported for IgA proteases from *N. gonorrhoeae* (M.H. Mulks, personal communication, *N. meningitidis* (Mulks *et al.*, 1980b; Plaut, 1983), and *H. influenzae* (Mulks *et al.*, 1980a; Kilian and Thomsen, 1983). Now that a reliable purification procedure for gonococcal IgA protease is available, a detailed analysis of the immunological and functional properties of variant IgA proteases can be accomplished. We believe that these studies will contribute to a clearer view of the role of IgA proteases in bacterial pathogenesis.

Materials and methods

Bacterial strains and growth media

N. gonorrhoeae MS11 was provided from the collection of E. C. Gotschlich. *E. coli* strains GC1 (K12, λ_{-m}^{+}) used for the transformation with hybrid DNA (Meyer *et al.*, 1982). DH1 (*recA*) used for the stable propagation and preparation of plasmid DNA (Hanahan, 1983), and DS410 (*minA*, *minB*) used for the preparation of minicells (Levy, 1974) have been described. GC agar base for growth of gonococci was purchased from BBL and supplemented with IsoVital X from the same vendor. To prepare IgA protease from gonococcal cultures a chemically defined medium described by Catlin (1973) was used and casamino medium (M9CA, see Maniatis *et al.*, 1982) was used for growth of *E. coli* cells to isolate plasmid DNA or IgA protease from culture supernatants.

Protein analysis

To prepare periplasmic fractions from *E. coli* by the osmotic shock procedure (Neu and Heppel, 1965), cells of a 3 ml GC1 (pIP1) culture were harvested and washed once in fresh culture medium. After the cells were suspended in 1 ml 20% sucrose in 1 mM EDTA and 30 mM Tris-Cl, pH 8 at 24°C, they were gently shaken for 10 min, collected, and resuspended in 0.5 ml ice cold water. The suspension was vigorously shaken for 10 min at 4°C, centrifuged to remove the cells, and the supernatant adjusted to 3 ml with casamino medium. The supernatant was tested for IgA protease and alkaline phosphatase activity as a marker enzyme in the periplasm. To obtain a complete cell lysate, cells from 3 ml of the same *E. coli* culture were collected, transferred to 3 ml cold culture medium, and sonicated in ice 6 times for 10 s with pauses of 30 s for cooling. IgA protease activity was determined by cleavage of labelled secretory IgA.

Protein concentrations were determined according to the method of Bradford (1976). Protein fractions were also analysed by polyacrylamide SDS gel electrophoresis (Laemmli, 1970) followed by the silver staining procedure of Switzer *et al.* (1979). The native mol. wt. of IgA protease was determined by gel filtration through Ultrogel AcA 44 (20 ml bed volume, 0.4 mm in diameter, equilibrated in 20 mM Tris-Cl (pH 8.1) and 300 mM NaCl and by sedimentation through a linear glycerol gradient [25–40% glycerol in 400 mM KCl, 20 mM Tris-Cl (pH 7.5), 5 mM β -mercaptoethanol, in a Beckman SW60Ti rotor at 58 000 r.p.m. and 4°C for 30 h]. 200 μ l fractions were collected in both cases. 10 μ l aliquots were tested for enzymatic activity, whereas the remainder of the fraction was precipitated with 5% TCA and analysed on a SDS polyacrylamide gel. This procedure allowed correlation between enzyme activity and protein composition. Ovalbumin (44 kd), bovine serum albumin (68 kd), and pancreatic alkaline phosphatase [140 kd, Boehringer Mannheim, assayed by the method of Garen and Levinthal (1960)] were used as marker protein in both experiments.

Filter assay for detection of IgA protease producing colonies

To test IgA protease activity we used secretory IgA purified from human colostrum (96% electrophoretic purity; Behring-Werke, Marburg). This preparation contained ~40% secretory IgA2 besides secretory IgA1. 25 μ g of

the immunoglobulin was iodinated at its tyrosine residues which are accumulated in the Fab portion, using the Chloramine T method of Hunter and Greenwood (1962) to a specific activity of $\sim 10^5$ c.p.m./mg with 125 I. This preparation was used either to measure IgA protease activity in soluble phase (see below) or to prepare nitrocellulose filters coated with labelled IgA on one side: nitrocellulose BA85 filters (Schleicher and Schüll) were sequentially incubated at 4°C on top of Whatman 3MM papers which were wetted with 150 mM NaCl and 50 mM Tris-Cl, pH 7.5 containing (a) 50 μ g/ml protein A (1 h), (b) 10 μ g/ml anti-IgA secretory component rabbit serum and 3% BSA (8 h), and (c) 2×10^5 c.p.m./ml [125 I]IgA and 3% BSA, for 2 h. Between each step the filters were washed twice in TBS solution and excess liquid was removed. After the last step the filters were sterilized by exposure to u.v. light. To check bacterial clones for IgA protease secretion, the IgA coated side of the filter was placed on top of an agar plate with freshly grown colonies (usually not larger than 1–2 mm in diameter). After incubation at 37°C for 4–10 h the filters were removed and washed twice in TBS. The dried filters were exposed to X-ray film for 12 h. Bacterial colonies releasing IgA protease could be detected on the autoradiograph as light spots corresponding to the positions at which labelled Fab fragments were removed by the specific proteolytic cleavage of IgA1 (see Figure 1).

Semi-quantitative test system for IgA protease

For the semi-quantitative determination of IgA protease activity, the assay mixture contained 10^5 c.p.m. radioactive IgA in 10 μ l casamino medium supplemented with chloramphenicol (100 μ g/ml). Incubation was carried out at 37°C after addition of cell culture supernatants or enzyme fractions to give 20 μ l total volume. 10 μ l of sample solution were added to the reaction mixture which was boiled for 5 min and then applied to a 12.5% acrylamide gel. After electrophoresis the gel was dried and autoradiographed. The uncleaved reaction product showed three major bands on the autoradiograph: secretory component (70 kd), heavy chain (65 kd), and light chain (30 kd). After complete digestion of IgA1 by IgA protease an additional band of ~35 kd, the strongly labelled N-terminal part of the IgA1 heavy chain, was seen. The corresponding C-terminal part was weakly labelled and usually not visible. The 65-kd band never completely disappeared after treatment with IgA protease, due to the remaining heavy chain of IgA2.

DNA isolation and manipulation

Chromosomal DNA from gonococcal strains was prepared from colonies grown on GC agar (Stern *et al.*, 1984). Plasmid DNA from *E. coli* cells was amplified by addition of 150 μ g/ml chloramphenicol to a logarithmically growing culture and isolated by alkaline lysis (Maniatis *et al.*, 1982).

For isolation of the IgA protease gene from *N. gonorrhoeae* MS11, 1 mg chromosomal DNA of a P^{-} variant was digested with *SalI* endonuclease and size-fractionated by sedimentation through a sucrose gradient [5–20% sucrose in 0.5 M NaCl, 50 mM Tris-Cl (pH 7.5), and 1 mM EDTA] at 20 000 r.p.m., 4°C for 12 h in a Beckman SW41 rotor. 0.5 ml fractions were precipitated by addition of 1 ml ethanol and aliquots were analyzed on agarose gels. The peak fraction, with a size distribution around 11 kb, was cut with *Clal* endonuclease and run through a second sucrose gradient. The peak fraction with a size of 9 kb, was ligated to a pBR322 derivative (named pBA and obtained from M. So), which carries a poly-linker insertion in the Tc resistance gene (see Bolivar *et al.*, 1977). This plasmid was cleaved with *Clal* and *SalI* and, in order to prevent self-ligation (see Meyer *et al.*, 1982), the smaller fragment was removed by sedimentation through sucrose. Ligated DNA was transformed into *E. coli* GC1 and selected for ampicillin resistance.

Physical analysis of DNA

For rapid analysis, plasmid DNA was isolated according to a slight modification of the procedure of Birnboim and Doly (1979). Plasmid DNA derived with this method from a primary transformant could be used for restriction analysis as well as for transformation of other *E. coli* strains.

Restriction enzymes were purchased from Boehringer Mannheim, New England Biolabs, and Renner. Restriction digestion of DNA, truncation of cloned inserts with *Bal31* exonuclease, and other general DNA technology procedures were performed as recommended in a recent laboratory manual (Maniatis *et al.*, 1982). Electrophoresis of DNA fragments was done in 0.8% (SeaKem ME) agarose horizontal gels in TBE buffer containing 100 mM Tris-borate pH 8.1 and 2.5 mM EDTA. Mol. wt. standards were phage λ HindIII fragments. Hybridization of chromosomal DNA with plasmid DNA was carried out essentially as described by Southern (1975) with minor specifications (Stern *et al.*, 1984).

Acknowledgements

We thank Ilge Wilhelm for excellent assistance in the cloning experiments. The project is supported by the Bundesministerium für Forschung und Technologie grant number BCT 0364 1.

References

- Birnboim, H.C. and Doly, J. (1979) *Nucleic Acids Res.*, **7**, 1513-1523.
- Bolivar, R., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L. and Boyer, H.W. (1977) *Gene*, **2**, 95-113.
- Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248-254.
- Bricker, J., Mulks, M.H., Plaut, A.G., Moxon, E.R. and Wright, A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2681-2685.
- Catlin, B.W. (1973) *J. Infect. Dis.*, **128**, 178-194.
- Frangione, B. and Wolfenstein-Todel, C. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 3673-3676.
- Garen, A. and Levinthal, C. (1960) *Biochim. Biophys. Acta*, **38**, 470-483.
- Goebel, W. and Hedgpeth, J. (1982) *J. Bacteriol.*, **151**, 1290-1298.
- Hanahan, D. (1983) *J. Mol. Biol.*, **166**, 557-580.
- Hunter, W.M. and Greenwood, F.C. (1962) *Nature*, **194**, 495-496.
- Kilian, M. and Thomsen, B. (1983) *Infect. Immun.*, **42**, 126-132.
- Koomey, J.M. and Falkow, S. (1984) *Infect. Immun.*, **43**, 101-107.
- Koomey, J.M., Gill, R.E. and Falkow, S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7881-7885.
- Kornfeld, S.J. and Plaut, A.G. (1981) *Rev. Infect. Dis.*, **3**, 521-534.
- Kratzin, H., Altevogt, P., Kortt, A., Ruban, E. and Hilschmann, N. (1978), *Hoppe-Seyler's Z. Physiol. Chem.*, **379**, 1717-1745.
- Laemmli, U.K. (1970) *Nature*, **227**, 680-685.
- Levy, S.B. (1974) *J. Bacteriol.*, **120**, 1451-1463.
- Maniatis, T., Fritsch, E.F. and Sambrook, J., eds. (1982) *Molecular Cloning, A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
- Meyer, T.F., Mlawer, N. and So, M. (1982) *Cell*, **30**, 45-52.
- Milazzo, F.M. and Delisle, G.J. (1984) *Infect. Immun.*, **43**, 11-13.
- Mulks, M.H., Kornfeld, S.J. and Plaut, A.G. (1980a) *J. Infect. Dis.*, **141**, 450-456.
- Mulks, M.H., Plaut, A.G., Feldman, H.A. and Frangione, B. (1980b) *J. Exp. Med.*, **152**, 1442-1447.
- Neu, H.C. and Heppel, L.A. (1965) *J. Biol. Chem.*, **240**, 3685-3692.
- Plaut, A.G. (1983) *Annu. Rev. Microbiol.*, **37**, 603-622.
- Plaut, A.G., Gilbert, J.V., Artenstein, M.S. and Capra, J.D. (1975) *Science (Wash.)*, **190**, 1103-1105.
- Plaut, A.G., Gilbert, J.V. and Wistar, R., Jr. (1977) *Infect. Immun.*, **17**, 130-135.
- Plaut, A.G., Gilbert, J.V. and Rule, A.H. (1978) in Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D. and Young, F.E. (eds.), *Immunobiology of Neisseria gonorrhoeae*, American Society for Microbiology, Washington, DC, pp. 279-284.
- Salit, I.E., Blake, M. and Gotschlich, E.C. (1980) *J. Exp. Med.*, **151**, 716-725.
- Southern, E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.
- Stern, A., Nickel, P., Meyer, T.F. and So, M. (1984) *Cell*, **37**, 447-456.
- Switzer, R.C., Merril, C.R. and Shifrin, S. (1979) *Anal. Biochem.*, **9**, 231-237.
- Williams, R.C. and Gibbons, R.J. (1972) *Science (Wash.)*, **177**, 697-699.

Received on 2 April 1984