Binding of Native α_2 -Macroglobulin to Human Group G Streptococci

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Binding of human α_2 -macroglobulin (α_2 M) to group G streptococci and to their immunoglobulin G (IgG)-binding proteins (protein G) was investigated. Native α_2 M bound specifically to strain G-148 with an apparent dissociation constant of $(2.2 \pm 1.5) \times 10^{-9}$ M. Proteinase-complexed α_2 M did not compete for the binding sites, and ^{125} I-labelled proteinase-complexed α_2 M did not bind to the bacteria. Binding of native α_2 M to the cells was not affected by IgG or protein G consisting of only IgG-binding domains. ^{125} I-labelled recombinant protein G did not bind to native or proteinase-complexed α_2 M. However, a lysate of G-148 cells inhibited binding of α_2 M to the bacteria, and immobilized wild-type protein G bound α_2 M directly from fresh human plasma. In 13 group G streptococcal isolates, IgG-binding proteins were immunologically identified as protein G. In 11 isolates, these molecules reacted also with α_2 M and human serum albumin (HSA). Western blots (immunoblots) of two wild-type protein G variants revealed identical bands reactive with goat IgG, HSA, and native α_2 M. Digestion of wild-type protein G with clostripain destroyed in both variants the binding sites for α_2 M but not for albumin and IgG. N-terminal fragments of protein G (lacking the IgG-binding region) bound both α_2 M and HSA, whereas a similar HSA-binding peptide lacking the first 80 amino acids did not react with α_2 M. Our findings are consistent with a specific binding site for native α_2 M in the N-terminal region of protein G and suggest that binding of α_2 M via IgG-binding proteins may be a general feature of human group G streptococci.

Human group G streptococci represent a part of the normal flora of the skin, pharynx, and female genital tract but have also been implicated in severe human infections (2, 30). Little is known about the mechanisms involved in group G streptococcal virulence. However, proteins structurally related to M proteins, the principal virulence factors of group A streptococci (19), have been identified in group G streptococcal strains (5, 14). Human group G streptococci also express receptors for plasma proteins like immunoglobulin G (IgG) (7), albumin (35, 40), fibronectin (29), plasminogen (49), and α_2 -macroglobulin (α_2 M) (31, 32). Among these, the IgG-binding protein, protein G (PG), has been extensively studied because of its practical value for immunochemical applications (9, 18). According to gene and protein sequence analysis, PG consists of repetitively arranged domains of which two to three highly homologous regions each have IgG-binding activity (17, 36). In PG from strain G-148, a region located N terminally to the IgG-binding site contains additional albumin-binding domains (1, 6). Strain G-148 also binds human α_2 M (32).

α_2 M is a large plasma glycoprotein (718 kDa) with several functions (8). As a broad-spectrum proteinase inhibitor, it inhibits serine, thiol, aspartic, and metallo-type proteinases (4). The native protein consists of four identical subunits (20, 46). The interaction between α_2 M and proteinases involves a conformational change of α_2 M, which results in an entrapment of the proteinase molecule. The conformational change of α_2 M leads to an exposure of recognition sites which may then interact with highly discriminating receptors on hepatocytes, macrophages, and fibroblasts (23, 50). In addition the conformational change of α_2 M reveals increased electrophoretic mobility in nondenaturing polyacrylamide gel electrophoresis (PAGE) and is therefore also called the fast form of α_2 M

(f- α_2 M). Consequently the native α_2 M molecule has been termed the slow form of α_2 M (s- α_2 M) (3).

Binding of α_2 M by streptococci is highly specific and conformation dependent. Bovine and equine group C streptococci bind the proteinase-complexed form of α_2 M, but human group G streptococci bind only native α_2 M (28, 32, 33). Little is known about the surface proteins responsible for the binding of native α_2 M to group G streptococci. However, it has been suggested that the IgG-binding repeats of PG from strain G-148 can also bind α_2 M, regardless of its conformation (45).

The aim of this study was to characterize the specificity of the α_2 M binding to group G streptococci and to identify the bacterial binding site. Our results show that like other human group G streptococci, strain G-148 binds exclusively the native form of human α_2 M with high specificity and affinity. Commercially available recombinant PG consistently failed to show any interaction with α_2 M in various assay systems. However, native full-length (wild-type) PG bound to α_2 M. Data which locate the α_2 M-binding site to the E region in the N-terminal portion of PG are presented.

MATERIALS AND METHODS

Plasma proteins. Human polyclonal IgG and human fibrinogen were obtained from Kabi AB (Stockholm, Sweden), human serum albumin (HSA) was obtained from Sigma (St. Louis, Mo.), bovine serum albumin (BSA; radioimmunoassay grade, Cohn fraction V) was obtained from United States Biochemical Corporation (Cleveland, Ohio), and human haptoglobin was obtained from Behringwerke (Marburg, Germany). Rabbit IgG and goat IgG were isolated by affinity chromatography on protein A (PA)-Sepharose and PG-Sepharose (Pharmacia, Uppsala, Sweden), respectively. ^{125} I-labelled mouse monoclonal IgG1 (RAST RIA reagent no. P 8324) was from Pharmacia Diagnostics (Uppsala, Sweden), and ^{125} I-labelled goat anti-mouse IgM was from Du Pont Scandinavia AB (Stockholm, Sweden). Human α_2 M was isolated from fresh plasma of one of the authors and processed on the day of bleeding. The purification scheme contained the initial steps of the method of Harpel (21) followed by chromatography on Sephacryl S-300 (Pharmacia) and Fractogel TSK AF-Blue (Merck, Darmstadt, Germany) as described previously (41). Quality checks of the α_2 M preparations with respect to homogeneity in sodium dodecyl sulfate (SDS)-PAGE, native PAGE, trypsin-binding activity, and exposure of receptor recognition sites were

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performed as described previously (41). Fibronectin was prepared from plasminogen-depleted bovine plasma via affinity chromatography on gelatin-Sepharcl 5-300 as described previously (27).

Antibodies. A hen was immunized 4 times at 2-week intervals subcutaneously with PG (Pharmacia) mixed with Freund's adjuvant (Sigma). Subsequently, the antibodies were purified from the egg yolks, and the crude preparation was affinity purified with a PG-Sepharose column.

Conjugates. HSA, α_2 M, and the hen antibody against PG were conjugated to horseradish peroxidase (HRPO) as previously described (41, 42). An affinity-purified goat anti rabbit IgG-HRPO conjugate was obtained from Bio-Rad (Richmond, Calif.).

PA and PG. PA was obtained from Pharmacia, and ^{125} I-labelled recombinant PG was obtained from Amersham Sweden (Solna, Sweden). According to the manufacturer, this PG preparation had a molecular weight of 35,000 and a specific activity of 12 mCi/mg. The two other recombinant PG preparations (one with an albumin-binding region and two IgG-binding regions and the other with three IgG-binding regions only) were a gift from B. Guss, Department of Microbiology, Swedish University of Agricultural Sciences. Wild-type PG was purchased from Calbiochem (catalog no. 539302; Calbiochem, La Jolla, Calif.) or isolated from strain G-148 as described below. Recombinant albumin-binding N-terminal fragments of PG were produced in *Escherichia coli* as described below.

Determination of protein concentrations. Purified plasma proteins in solution were spectrophotometrically determined at 280 nm, assuming values of $A_{280} = 1.38$ for 1 mg of IgG per ml (15), $A_{280} = 0.90$ for 1 mg of human α_2 M per ml (20), $A_{280} = 0.67$ for 1 mg of BSA per ml, and $A_{280} = 0.53$ for 1 mg of HSA per ml (38). Solutions of PA and PG were prepared from lyophilized proteins and adjusted gravimetrically. Concentrations of recombinant PG with three IgG-binding regions were calculated with a molecular weight of 30,000 as deduced from the amino acid sequence (16).

Bacteria and growth conditions. Group G streptococcal strain G-148 was originally kindly provided by E. B. Myhre, Lund, Sweden, and clinical isolates of group G streptococci were provided by P. Kuusela, Department of Microbiology and Immunology, University of Helsinki, Helsinki, Finland. Lyophilized cells of *Streptococcus agalactiae* NCTC 8181 served as negative control cells in binding assays with ^{125} I-labelled proteins. The streptococci were grown in Todd-Hewitt broth (Merck) supplemented with 0.3% yeast extract for 18 h at 37°C.

Guanidinium hydrochloride treatment of streptococci. For binding studies, the cells were harvested by centrifugation ($20,000 \times g$, 20 min, 8°C), washed once with phosphate-buffered saline (PBS; pH 7.4), and resuspended in PBS (pH 7.4) containing 6 M guanidinium hydrochloride, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Merck), and 0.05% NaN_3 . After being stirred overnight at 4°C, the cells were collected by centrifugation, washed three times with PBS (pH 7.4), and either stored as a suspension in PBS at -20°C or washed once more with distilled water and lyophilized. The purpose of the extraction was stabilization of the bacterial cells by removal of proteolytic and cell wall-degrading enzymes and possible extracellular slime material (42).

Binding and inhibition assays. Plasma proteins were labelled by the chloramine T method as described previously (43). Stock solutions of 5 $\mu\text{g}/\text{ml}$ (with specific activities of 3.4 to 9 mCi/mg of ligand) were stabilized by 1 mg of BSA per ml. For binding assays, a 1-mg/ml suspension of lyophilized bacteria was prepared in PBS with 0.1% BSA and mixed for 15 s in an ultrasonic bath (Bransonic 32; Branson, Danbury, Conn.). Aliquots of 300 μl were transferred to Eppendorf tubes containing 100 μl of PBS-BSA and 100 μl of the labelled ligand (usually diluted 1:50 in PBS-BSA). Proteins serving as potential inhibitors were twofold serially diluted in PBS-BSA, and 100 μl of each dilution was added to the tubes instead of the 100 μl of PBS-BSA. After 2 h incubation at room temperature, the cells were washed twice with PBS containing 0.05% Tween 20 (PBS-T). Subsequently, the radioactivity associated with the bacteria (counts per minute) was measured in a gamma counter (LKB-Wallac, Turku, Finland). Data from inhibition assays with unlabelled α_2 M were subjected to nonlinear least-squares analysis of binding data, using the Ligand program (34).

Analysis of proteins bound to PG-Sepharose. PG-Sepharose Fast Flow (Pharmacia) was filled into small plastic columns (empty NAP 5 columns; Pharmacia) to form a 1-ml gelbet. The columns were equilibrated with PBS containing 0.5% BSA and then loaded with 100 μl of ^{125} I-labelled human s- α_2 M, f- α_2 M (62,000 cpm of each), or monoclonal murine IgG1 (84,000 cpm). The samples were allowed to drain into the gelbet, and the columns were washed with 20 ml of PBS containing 0.5% BSA. The radioactivity (counts per minute) remaining associated with the gels was determined in a gamma counter (LKB-Wallac) and expressed as percentage of the added radioactivity.

Analysis of binding of ^{125} I-labelled PG to immobilized plasma proteins. Serial twofold dilutions of human, goat, and rabbit IgG, human α_2 M, and HSA were prepared in PBS starting with 100 μg of each protein per ml. Then 100 μl of each dilution was applied to a nitrocellulose sheet in a slot blot device (Bio-Rad). The nitrocellulose was saturated for 2 h in PBS containing 0.1% (vol/vol) Tween 20 at room temperature. Subsequently, the sheet was incubated overnight with ^{125} I-PG (200,000 cpm/ml in PBS-T). Autoradiography was performed for 2 days at -70°C, using Hyperfilm type MP (Amersham).

Release of the α_2 M-binding protein. Guanidinium hydrochloride-treated and lyophilized streptococci were suspended in 0.025 M Tris-0.14 M NaCl (pH 7.4) containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (100 mg [dry

weight] of cells per 10 ml of buffer). The suspension was vigorously agitated in an ultrasonic water bath (Bransonic 32) for 10 min, the cells were then removed by centrifugation ($20,000 \times g$, 20 min), and the clear supernatant ($A_{280} = 0.650$) was collected and either used directly in inhibition assays or analyzed after five- to sixfold concentration (Millipore ultrafree-MC filter unit with a type PLGC membrane; Millipore, Bedford, Mass.) by SDS-PAGE.

Isolation of wild-type PG. Group G streptococci were grown overnight in Todd-Hewitt broth supplemented with 0.6% (wt/vol) yeast extract and 10 mM glycine. Two hours before harvesting, the culture medium was adjusted to 0.68 M glycine. Cells obtained after centrifugation were washed twice in 50 mM Tris-50 mM EDTA (pH 7.5) containing 0.025% NaN_3 and suspended in 50 mM Tris-50 mM EDTA (pH 7.5) containing 25% sucrose. The suspension was then incubated with 30 mg of hen egg white lysozyme (Boehringer Mannheim, Mannheim, Germany) per ml for 1 week at 4°C. Aliquots were withdrawn in 2-day intervals for monitoring of released proteins. Solubilized PG was detected both by Western blotting (immunoblotting) and by a sandwich enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with 5 μg of human or caprine IgG per ml for adsorption of PG and HRPO-conjugated HSA for detection of bound PG. The bound PG was also tested for binding of an α_2 M-HRPO conjugate. After addition of the chromogenic substrate 3,3',5,5'-tetramethylbenzidine, the plates were measured with an ELISA reader (type iEMS; Labsystems, Helsinki, Finland). The cell-free supernatant of strain G-148 obtained after centrifugation was also adjusted to 0.5 M NaCl and affinity purified on a 1-ml column of HSA-Sepharose. The column was washed with PBS (pH 7.5) and eluted with 0.3 M acetic acid adjusted to pH 2.8. The elution was recorded at 280 nm.

Cleavage of wild-type PG with clostripain. PG was digested with clostripain (sequencing grade; Promega, Madison, Wis.) in 10 mM Tris (pH 7.4)-1 mM CaCl_2 -2 mM dithiothreitol at an enzyme/substrate ratio of 1:50. Digestion was terminated after 4 h of incubation at 37°C by addition of EDTA (final concentration, 5 mM).

Production and purification of cloned fragments of PG. *E. coli* strains and plasmids were kindly provided by Åke Nygren, Royal Institute of Technology, Stockholm, Sweden. *E. coli* TG1 harboring plasmid pB1B2T encoded a ca. 26-kDa protein (designated B1B2) consisting of the albumin-binding region of PG, and *E. coli* RR1ΔM15 containing plasmid pSPG5' encoded a ca. 35-kDa protein (designated EB1B2) consisting of the N-terminal portion of PG with the Ss, E, A1, B1, A2, B2, A3, and S regions (nomenclature according to Olsson et al. [36]). The strains were grown overnight in Luria-Bertani medium supplemented with 0.6% (wt/vol) yeast extract and 150 μg of ampicillin per liter. Cells were harvested by centrifugation and subsequently lysed on ice for 60 min in 50 ml of lysis buffer, consisting of 10 mM Na_2HPO_4 , 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mg of hen egg white lysozyme (Boehringer Mannheim) per ml. The lysates were extensively sonicated on ice for 2 min to reduce the viscosity and centrifuged for 30 min at $20,000 \times g$. The clear supernatants were diluted with equal volumes of PBS containing 0.05% Tween 20 and 0.05% NaN_3 . The samples were then passed through a 1-ml HSA-Sepharose column at a flow rate of 2 ml/min. After extensive washings with the start buffer, the column was eluted with 0.3 M acetic acid adjusted to pH 2.8.

SDS-PAGE and Western blotting. For analysis of bacterial extracts as well as of the proteins serving as free ligands or inhibitors, a Phast system electrophoresis unit (Pharmacia) with gradient gels was used. SDS-PAGE was also run in a Mini-protein chamber (Bio-Rad). Phast system gels were diffusion blotted at 60°C for 30 min. Electrophoretic blotting of the minigels was performed in the buffer system described by Towbin et al. (47) under constant-current conditions (200 mA) for 2 h. After blotting, the nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) were saturated with 0.1% Tween 20 in PBS. IgG-binding proteins were detected by incubating the sheets with a goat anti-rabbit IgG-HRPO conjugate diluted 1:3,000. After washing, reactive bands were detected with 4-chloro-1-naphthol (Serva).

Fluorescent assay for direct binding of α_2 M from human plasma. Microtiter plates (white Maxisorp; Nunc, Denmark) were coated either with PG (wild type, purified on HSA-Sepharose) or with Mal-A2M-R (5 $\mu\text{g}/\text{ml}$), a fusion protein between the maltose-binding protein from *E. coli* and the N-terminal region of the type III Fc receptor from *Streptococcus dysgalactiae* Sc1 (26) which binds f- α_2 M. After overnight incubation at 4°C, the plates were washed and then blocked for 1 h with PBS-T. Serial twofold dilutions of fresh human EDTA-plasma or trypsin-treated plasma were applied to the wells (100 μl per well), starting with a dilution of 1:50. The plasma was allowed to react for 3 h, the plate was washed, and the wells previously incubated with native plasma were treated with 100 μl of trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK] treated; 33 $\mu\text{g}/\text{ml}$ in PBS; Merck); after 5 min, 50 μl of soybean trypsin inhibitor (solution of 100 $\mu\text{g}/\text{ml}$ in PBS; Merck) was added. Wells previously incubated with trypsin-treated plasma were filled with PBS (150 μl per well). Finally, 50 μl of the fluorogenic substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin-acetate (Bachem, Bubendorf, Switzerland) was added to all wells to give a final concentration of 0.1 M, and the plate was measured with a Fluoroscan II reader (Labsystems).

Microtiter plate assay for inhibition of α_2 M binding to cells by fragments of PG. White Maxisorp plates (Nunc) were coated with 200 μg of G-148 cells per ml overnight at room temperature and subsequently blocked for 1 h at room temperature with PBS containing 0.1% Tween 20. Recombinant PG prepara-

FIG. 1. Inhibition of binding of ^{125} I-labelled native α_2 M to group G streptococcal strain G-148 by unlabelled native and proteinase-complexed α_2 M. Native α_2 M (●) and trypsin-complexed α_2 M (α_2 M-T; □) were mixed with ^{125} I-labelled native α_2 M before addition to bacterial suspensions. The radioactivity associated with the bacterial cells after 1 h of incubation and subsequent washings was determined in a gamma counter. Data (representing the means of duplicate determinations) were plotted as percentage of α_2 M binding in the absence of inhibitory proteins (100% corresponding to 24,000 cpm).

tions, one with three IgG-binding regions only and another with one albumin-binding region and two IgG-binding regions, and protein EB1B2, representing the N-terminal portion of PG with albumin- but not IgG-binding regions, were twofold serially diluted, starting with a concentration of 400 μ g/ml. Subsequently, 50 μ l of each dilution was added to the wells, after which 50 μ l of 1:8,000-diluted native α_2 M-HRPO conjugate was added. After 6 h of incubation, the plates were washed, and bound α_2 M was detected with the fluorogenic substrate 3-*p*-hydroxyphenylpropionic acid (Sigma) as described previously (48).

RESULTS

Specificity of the binding assay. Binding of human ^{125} I-s- α_2 M increased with the amount of G-148 cells present in the binding assay but not with increasing amounts of group B streptococcal cells (strain NCTC 8181), serving as a control. Even at the highest concentration of this strain (800 μ g [dry weight] per ml), the remaining radioactivity associated with the bacteria did not exceed 0.63% of the added radioactivity.

Dissociation constants and conformation specificity of α_2 M binding. The binding of ^{125} I- α_2 M to strain G-148 was effectively inhibited by excess of unlabelled s- α_2 M. On the contrary, high concentrations (up to 200 nM) of trypsin-treated f- α_2 M did not show any inhibitory effect on the binding of ^{125} I- α_2 M to cells of strain G-148, and a concentration of 900 nM f- α_2 M caused only a 10% reduction of the bound tracer α_2 M (Fig. 1). To verify this observation, the ^{125} I-s- α_2 M was also treated with trypsin before application in the binding assay. Electrophoretic analysis confirmed that the ^{125} I-s- α_2 M had been completely converted to the fast form. The same amount of bacteria that bound 66% of the ^{125} I-s- α_2 M bound less than 1% of the f- α_2 M. Data from the saturation isotherms obtained with two different s- α_2 M preparations under otherwise identical conditions were analyzed by nonlinear least-squares curve fitting. Best fits were obtained for models of one type of binding site. For the freshly isolated highly purified s- α_2 M preparation, a K_D of $(2.2 \pm 0.15) \times 10^{-9}$ M was determined. A slightly higher K_D of $(4.4 \pm 0.53) \times 10^{-9}$ was calculated when an α_2 M preparation that had been frozen once was used.

Binding of α_2 M to G-148 in the presence of IgG or PG. No reduction of the s- α_2 M binding was observed in the presence of recombinant PG (with three IgG-binding regions) in up to 360,000-fold molar excess over α_2 M. Similarly, increasing amounts of IgG in concentrations up to 2.2 mM corresponding

to a 70,000-fold molar excess over α_2 M did not show any influence on the binding of the labelled ligand. Likewise, other plasma proteins, including haptoglobin, fibrinogen, fibronectin, and HSA, did not influence the binding of α_2 M to G-148 (data not shown).

Binding of s- α_2 M, f- α_2 M, and IgG to PG-Sepharose and to G-148 cells. To further elucidate the potential role of PG in α_2 M-binding, ^{125} I-labelled α_2 M, both in the native slow form and in the converted (trypsin-treated) fast form, as well as ^{125} I-IgG were passed through small PG-Sepharose columns. None of the α_2 M samples were retained to more than 1%, whereas 59% of the IgG preparation was bound. When cells of strain G-148 were allowed to react with the same protein preparations in direct binding assays, 58% of human s- α_2 M but only 1% of the f- α_2 M associated with the cells. Of the IgG preparation, 11% was bound to the bacteria.

Binding of ^{125} I-protein G to α_2 M, IgG, and albumin in slot blots. In agreement with all other assays of this study, the only immobilized plasma proteins observed to bind ^{125} I-labelled recombinant PG were the IgG preparations of human, rabbit, and goat origin; the α_2 M preparations did not show any signal (data not shown).

Inhibitory activity of a crude ultrasonic extract from G-148 cells. To identify the component responsible for binding of s- α_2 M to G-148, a crude G-148 cell extract obtained by ultrasonication was tested for inhibitory activity. Addition of 100 μ l of extract to the binding assay inhibited α_2 M binding more than 70%. When the bacteria were subsequently washed and reincubated with ^{125} I-s- α_2 M in the absence of the cell extract, they retained their original binding capacity, thus excluding the possibility that the inhibition observed before was due to destruction of sensitive bacterial binding sites by potential proteolytic activity in the extract. Cells of *S. dysgalactiae* Sc1 (which specifically bind f- α_2 M) were included as control for the conformation of α_2 M. Binding to these cells did not amount to more than 10% of the added radioactivity, and preincubation of the labelled s- α_2 M with the G-148 extract did not increase binding to Sc1, thus excluding a potential conversion of α_2 M to the fast form by components in the extract.

Analysis of the crude extract by Western blotting. The concentrated ultrasonic extract was subjected to SDS-PAGE and blotted to nitrocellulose sheets. For comparison, samples of PA and two preparations of recombinant PG, one containing three IgG-binding regions only and the other containing also the albumin-binding site, were run in parallel. All IgG-binding preparations and the crude extract reacted with an affinity-purified goat anti-rabbit IgG-HRPO conjugate, as expected. Subsequently, the blot was probed with ^{125} I- α_2 M. Only the crude extract of G-148 revealed distinct signals. The PA and PG samples in concentrations apparently even higher than in the crude extract did not show any reaction with ^{125} I- α_2 M even after overexposure of the X-ray film.

Occurrence of α_2 M and IgG receptors on other human group G streptococci. Fourteen beta-hemolytic clinical isolates of group G streptococci were compared with strain G-148 in binding assays using ^{125} I-s- α_2 M and ^{125} I-labelled goat IgG as the ligand (Fig. 2). While all isolates bound IgG in the range of 30 to 81%, 3 of the 14 isolates did not bind s- α_2 M. In control assays using ^{125} I-f- α_2 M as the ligand, none of the tested bacteria revealed significant activity (data not shown). Additionally, binding of s- α_2 M was also measured in the presence of 100 μ g of human IgG, corresponding to an 8,000-fold molar excess of IgG over α_2 M. The averages of α_2 M binding to the positive strains in the absence or presence of IgG were determined to be 77.8 and 78.4%, respectively, thus indicating no significant influence of the IgG on binding of α_2 M to the bacteria.

FIG. 2. Binding of ^{125}I -labelled native $\alpha_2\text{M}$ and goat IgG to clinical isolates of human group G streptococci. Filled bars, binding of $\alpha_2\text{M}$; open bars, binding of IgG. Binding is expressed as percentage of the total radioactivity added (100% corresponding to $\sim 20,000$ cpm).

Identification of native PG as the $\alpha_2\text{M}$ -binding protein.

While the above-described experiments with recombinant PG (truncated N terminally to the IgG-binding regions) excluded the possibility that the IgG-binding sites in PG were involved in $\alpha_2\text{M}$ -binding, the data for group G streptococcal clinical isolates indicated a correlation between IgG and $\alpha_2\text{M}$ binding. This finding suggested that other regions on the native PG molecule could still be responsible for the $\alpha_2\text{M}$ binding. To investigate this possibility, two native PG preparations were obtained: a commercial preparation from Calbiochem and another isolated from strain G-148 cells as described in Materials and Methods. As shown in Fig. 3, native PG immobilized on microtiter plates bound s- $\alpha_2\text{M}$ directly from fresh human EDTA-plasma. The recombinant $\alpha_2\text{M}$ -binding protein from protein MIG, a type III Fc receptor from *S. dysgalactiae* with N-terminal $\alpha_2\text{M}$ -binding activity (26), failed to absorb s- $\alpha_2\text{M}$ from fresh human plasma, in accordance to its known specificity for f- $\alpha_2\text{M}$. In contrast, trypsin-treated plasma resulted in strong binding to protein MIG and complete failure to interact with PG. In addition, cell-free supernatants of lysozyme lysates of the 14 group G streptococcal isolates (Fig. 2) were allowed to react with IgG-coated microtiter wells. Subsequent probing of the plates for albumin-binding and $\alpha_2\text{M}$ -binding activity

FIG. 3. Direct binding of $\alpha_2\text{M}$ from fresh human plasma to purified wild-type PG (●) and to protein MIG, the $\alpha_2\text{M}$ - and IgG-binding protein from *S. dysgalactiae* (□). Serially twofold dilutions of fresh human plasma (native plasma) or of trypsin-treated plasma (T-plasma) were applied to microtiter wells coated with the streptococcal proteins. Bound $\alpha_2\text{M}$ was determined by an $\alpha_2\text{M}$ -specific assay based on the entrapment of trypsin (see Materials and Methods for details). The trypsin activity associated with the wells was measured by using a fluorogenic substrate.

FIG. 4. Reactions of human $\alpha_2\text{M}$, HSA, and IgG with wild-type PG in Western blots. Affinity-purified PG from a lysate of the group G streptococcal strain G-148 (G-148) and PG purchased from Calbiochem (CBC) were separated by SDS-PAGE (8 to 25% polyacrylamide gel) and blotted to nitrocellulose. Samples were either untreated (lanes A) or digested with clostripain (lanes B) before SDS-PAGE. The nitrocellulose strips were probed with goat IgG, HSA, and $\alpha_2\text{M}$ (all conjugated to HRPO). Numbers at the left indicate the relative positions of marker proteins in kilodaltons. Coomassie-stained gels (stain) with the respective protein samples are also shown.

revealed that 11 of 14 strains previously identified to bind $\alpha_2\text{M}$ all expressed trifunctional molecules, binding to IgG, HSA, and $\alpha_2\text{M}$. Furthermore, 13 of the isolates reacted with hen antibodies which had been raised against the IgG-binding part of PG and had been subsequently affinity purified on PG-Sepharose and conjugated to HRPO. Wild-type PG from Calbiochem and PG from strain G-148 were also subjected to Western blotting and subsequently probed with HRPO conjugates of HSA, s- $\alpha_2\text{M}$, and goat anti-rabbit IgG. Both bacterial proteins reacted strongly with all three conjugates (Fig. 4, lanes A).

To further localize the $\alpha_2\text{M}$ -binding activity within the PG molecule, both PG preparations were digested with clostripain. Eight cleavage sites were predicted for this enzyme within the PG molecule by the PC-Gen software package, of which seven were located N terminally to the IgG-binding region of PG. No cleavage site was located within the IgG-binding region. Under the conditions used for cleavage, the PG apparently lost its $\alpha_2\text{M}$ -binding activity and showed reduced HSA binding, whereas the IgG-binding activity remained unaffected, as judged from Western blots (Fig. 4, lanes B).

Expression of recombinant PG fragments and probing for $\alpha_2\text{M}$ -binding activity. Since the IgG-binding region of PG was

FIG. 5. Schematic drawing of PG and the different domains. Products of subcloned gene fragments are given below. Cleavage sites predicted for clostripain are indicated by arrows. SPG, PG with the signal sequence Ss. The albumin-binding regions A and B, the IgG-binding regions C1, C2, and C3, the wall-spanning region W, and the membrane-anchoring region M (nomenclature according to Ohlsson et al. [36]) are indicated. B2C1C3 is an albumin- and IgG-binding recombinant fragment, EB1B2 represents the N-terminal region of PG truncated C terminally in the IgG-binding repeats, and B1B2 represents an albumin-binding fragment.

found consistently in all assays not to be involved in α_2 M binding, protein B1B2, representing the albumin-binding region of PG, and protein EB1B2, representing the entire N-terminal portion of PG including the albumin-binding but not the IgG-binding region (Fig. 5), were separately purified from the respective *E. coli* clones and analyzed in Western blots. As shown in Fig. 6, the recombinant protein including the N-terminal but not the albumin-binding region alone was able to bind human α_2 M. Thus, the α_2 M-binding site could be localized within the alanine-rich E region of PG (Fig. 5).

Inhibition of α_2 M binding to whole cells of strain G-148 by PG fragments. To further evaluate the importance of the N-terminal region of PG for the interaction with α_2 M, binding of human s- α_2 M to whole cells of strain G-148 was studied in a microtiter plate assay using the recombinant PG-derived proteins as potential inhibitors. The protein representing the IgG-binding region as well as the protein representing the HSA-binding region (protein B1B2) had no effect in this binding assay. The protein representing the N-terminal region of PG and the adjacent albumin-binding region (protein EB1B2), however, completely inhibited the interaction between s- α_2 M and the streptococcal cells (Fig. 7).

DISCUSSION

α_2 M is a predominant proteinase inhibitor of the plasma but is found in lower concentrations also in other body fluids and secretions (10, 24). At present, proteinase inhibition is the

FIG. 6. Binding of HRPO-conjugated human α_2 M and albumin to recombinant PG fragments and wild-type PG. The recombinant PG fragments represented by protein EB1B2 (lanes 1) and protein B1B2 (lanes 2) (see Fig. 5) were produced in *E. coli* and affinity purified on HSA-Sepharose. The wild-type PG (lanes 3) had been isolated from a lysozyme lysate of strain G-148. Samples were subjected to SDS-PAGE and Coomassie staining (Stain) or to Western blotting and then probed with an HSA- or human α_2 M-HRPO conjugate. The positions (in kilodaltons) of molecular mass markers are indicated on the left.

best-understood function of α_2 M. Nevertheless, it is becoming increasingly clear that α_2 M participates also in immunoregulation (8). Interactions of cytokines with α_2 M in inflammatory processes are being intensively studied, and the interaction between α_2 M and pathogenic bacteria may reveal an additional role of α_2 M in bacterial infections.

In this investigation, using highly purified α_2 M in a defined conformation, we demonstrated high-affinity binding sites for native human α_2 M on the group G streptococcal strain G-148. The specificity for the native form of α_2 M differentiates, in accordance with an earlier report (32), the human group G streptococci from animal group C streptococci, which show selective specificity for the converted form of α_2 M.

An earlier study on the interaction of α_2 M with group G streptococci did not consider different conformations of α_2 M (12). The lyophilized α_2 M preparation used in those studies had been only about 60% active with respect to proteinase inhibition (30a) and thus most likely represented a mixed population of both conformations. In the present investigation using native α_2 M, a significantly higher affinity for the binding of s- α_2 M to group G streptococci was observed. However, it cannot be excluded that this difference was due to variations in the receptor structures of different strains.

FIG. 7. Inhibition of native α_2 M binding to strain G-148 cells by recombinant, PG-derived fragments. Twofold serial dilutions of protein EB1B2 (the N-terminal fragment of protein G; ●), protein B1B2 (an albumin-binding fragment; □), and protein B2C1C2 (representing an albumin- and IgG-binding fragment; △) were mixed with equal volumes of a human α_2 M-HRPO conjugate (prediluted 1:8,000 in PBS-Tween). One hundred microliters of each dilution step was added in duplicate to the microtiter wells coated with G-148 cells and allowed to react for 4 h. Fluorescent signals obtained with a fluorogenic substrate were then recorded with a Fluoroscan reader.

Conflicting results have been published with respect to the specificity of the α_2 M-streptococcus interaction. While earlier studies did not show any influence of IgG on the binding of α_2 M to group G streptococci (12, 32), more recent data indicated that higher concentrations of human IgG may inhibit the binding of α_2 M to group G streptococcal strain G-148. Moreover, it was reported that the IgG-binding region of PG (derived from strain G-148) was responsible for binding of both IgG and α_2 M (45).

In contrast, our data show that the binding of α_2 M to group G streptococci occurs independently of the IgG-binding site of PG. Thus, high concentrations of neither IgG nor recombinant PG in the competitive binding assays showed any influence on the binding of ^{125} I-s- α_2 M to strain G-148. Similar results were obtained when a number of clinical group G streptococcal isolates were tested for s- α_2 M binding. Likewise, by passing ^{125} I-labelled α_2 M through a PG-Sepharose column, practically all radioactivity was obtained in the flowthrough. While these experiments were performed with α_2 M in solution, the large inhibitor molecule could possibly change its conformation when immobilized on a solid surface and thereby express altered binding properties. We have also evaluated this possibility by immobilizing α_2 M (native and trypsin treated) as well as IgG preparations in various concentrations on nitrocellulose. The subsequent incubation with ^{125} I-labelled recombinant PG (truncated N terminally to the IgG-binding regions) revealed again interactions only with the IgG preparations. Thus, our findings are in agreement with earlier reports (12, 28, 32) stating that α_2 M binds to specific sites on the streptococcal surface independently of IgG-binding sites.

However, by using two wild-type preparations of PG, one from strain G-148 and another a commercial product, it could be shown that α_2 M binding was actually mediated by the N-terminal portion of PG, a region that in most commercially available PG products had been truncated since it also contains an albumin-binding site (1, 6).

Binding of s- α_2 M to group G streptococci has been reported to be extremely trypsin sensitive (32). In this study, digestion of wild-type PG with the more specific enzyme clostripain resulted in loss of the s- α_2 M-binding activity but not of albumin- and IgG-binding activity. Sequence analysis of PG for clostripain and trypsin cleavage sites revealed that both proteases could cleave within the N-terminal E region (two and six times, respectively). Comparison of two recombinant N-terminal fragments of PG (one with the complete N-terminal region and another one lacking the E region) demonstrated that the E region was responsible for binding of s- α_2 M.

According to our results, interaction of s- α_2 M with bacterial cells or with immobilized wild-type PG is not likely to lead to a true conformational change of the α_2 M molecule. However, such an effect has been reported to occur with an s- α_2 M-binding protein from group A streptococci (11). There are essentially two observations which argue against a conformational change upon reaction of α_2 M with the group G streptococcal α_2 M-binding protein. First, incubation of native α_2 M with cell wall extracts of strain G-148 (containing native PG) did not lead to increased binding of α_2 M to *S. dysgalactiae* Scl as would be expected if the f- α_2 M had been generated (11, 28, 32, 41). Second, α_2 M bound to the surface of group G streptococci or to immobilized PG could be quantitated by the entrapment of trypsin in such a way that its proteolytic activity was retained in the presence of soybean trypsin inhibitor. This reaction would be impaired if the α_2 M had been transformed to its inactive fast form.

Our results indicate that native α_2 M (capable of trypsin binding) may become deposited on the surface of human

group G streptococci by strongly binding to the N-terminal region of PG. The experiments with a limited number of group G streptococcal isolates suggest that binding of native α_2 M via IgG-binding molecules may be a common feature of human group G streptococci. Thus, the IgG-binding molecules obtained from the 11 isolates positive for α_2 M binding all bound α_2 M and albumin, and they were also recognized by a hen antibody raised against PG. Furthermore, the two analyzed wild-type PG preparations, both binding α_2 M, varied significantly in size (67 and 57 kDa in SDS-PAGE) and in the size of the IgG-binding fragments obtained by clostripain cleavage, which indicates their separate origins.

PG molecules of various strains have been compared by sequence and found to be highly similar in IgG-binding regions and similar to a lesser extent in albumin-binding regions, differing in the number of repeat units (37, 44). We have recently described two IgG-binding proteins from *S. dysgalactiae* which additionally bind α_2 M (protein MIG) and α_2 M and albumin (protein MAG) (25, 26). While the IgG-binding regions in protein MIG and protein MAG, the albumin-binding region in protein MAG, and the C-terminal cell wall-spanning and membrane-anchoring regions are highly similar to corresponding regions of PG, the N-terminally located α_2 M-binding regions in both MIG and MAG are unique, with no homology to the corresponding N-terminal region of PG or to each other. Moreover and in contrast to PG, the binding of α_2 M to these proteins requires the proteinase-complexed form of α_2 M (f- α_2 M).

Taken together, the reported findings indicate that binding of the proteinase inhibitor α_2 M may be a common, novel function of the N termini of many IgG-binding surface proteins of streptococci. Although little is known about the role of IgG-binding proteins in virulence, it is clear that their contribution can be understood only if the multifunctional properties of the molecules are considered. In the past, IgG-binding proteins from streptococci have been studied primarily as biochemical tools (13). More recent studies of IgG-binding proteins associated with pathogenic group A streptococci have raised an interest in their role in pathogenesis (13, 39). Genetic analyses have revealed surprising similarities between IgG-binding proteins and the antiphagocytic M proteins of group A streptococci. It is becoming clear that both proteins are functionally similar variants of a common ancestral protein (13, 22). As α_2 M-binding activity has also been found in group A streptococci, it will be interesting to investigate whether this interaction is also mediated by IgG-binding proteins. Further studies of the mechanism of α_2 M binding are required to elucidate the role of α_2 M binding in streptococcal infections.

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