M1 protein and Protein H: IgGFc- and albumin-binding streptococcal surface proteins encoded by adjacent genes

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M1 protein and Protein H are surface proteins simultaneously present at the surface of certain strains of *Streptococcus pyogenes*, important pathogenic bacteria in humans. The present study concerns the structure, protein-binding properties and relationship between these two molecules. The gene encoding M1 protein (*emm1*) was found immediately upstream of the Protein H gene (*sph*). Both genes were preceded by a promoter region. Comparison of the sequences revealed a high degree of similarity in the signal peptides, the C repeats located in the central parts of the molecules and in the C-terminal cell-wall-attached regions, whereas the N-terminal sequences showed no significant similarity. Protein H has affinity for the Fc region of IgG antibodies. Also M1 protein, isolated from streptococcal culture supernatants or from *Escherichia coli* expressing *emm1*, was found to bind human IgGFc. When tested against polyclonal IgG from eight other mammalian species, M1 protein and Protein H both showed affinity for baboon, rabbit and pig IgG. M1 protein also reacted with guinea-pig IgG, whereas both streptococcal proteins

were negative in binding experiments with rat, mouse, bovine and horse IgG. The two proteins were also tested against other members of the immunoglobulin super family: human IgM, IgA, IgD, IgE, β_2 -microglobulin, and major histocompatibility complex (MHC) class-I and class-II antigens. M1 protein showed no affinity for any of these molecules whereas Protein H reacted with MHC class-II antigens. M1 protein is known to bind albumin and fibrinogen also. The binding sites for these two plasma proteins and for IgGFc were mapped to different sites on M1 protein. Thus albumin bound to the C repeats and IgGFc to a region (S) immediately N-terminal of the C repeats. Finally, fibrinogen bound further towards the N-terminus but close to the IgGFc-binding site. On the fibrinogen molecule, fragment D was found to mediate binding to M1 protein. The IgGFc-binding region of M1 protein showed no similarity to that of Protein H. Still, competitive binding experiments demonstrated that the two streptococcal proteins bound to overlapping sites on IgGFc.

INTRODUCTION

Streptococcus pyogenes are bacteria responsible for a number of suppurative infections in humans, such as acute pharyngitis and skin infections, and rheumatic fever and glomerulonephritis are serious complications that can follow infections with this micro-organism. Different strains of *S. pyogenes* express surface molecules, M proteins, of more than 80 different serotypes which are regarded as major virulence factors because of their anti-phagocytic property [for references see Fischetti (1989)]. M proteins are α -helical coiled-coil hair-like molecules on the streptococcal surface (Phillips et al., 1981). Other surface proteins, with affinity for IgG and/or IgA, have more recently been isolated and characterized from different strains of *S. pyogenes* (Grubb et al., 1982; Yarnall and Boyle, 1986; Heath and Cleary, 1987; Lindahl and Åkerström, 1989; Åkesson et al., 1990; Bessen and Fischetti, 1992; Stenberg et al., 1992). These proteins show considerable similarity to each other and to M proteins in certain regions but they also have unique sequences. The same streptococcal strain can express both an M protein and an immunoglobulin-binding protein, and the corresponding genes are often closely linked on the streptococcal chromosome (Heath and Cleary, 1987; Lindahl, 1989; Bessen and Fischetti, 1992; Haanes et al., 1992; Stenberg et al., 1992). In contrast with M proteins, immunoglobulin-binding streptococcal proteins have not been shown to play a role in pathogenicity or virulence. However, a large majority of clinical isolates of *S. pyogenes* do express immunoglobulin-binding surface proteins (Lindahl and Stenberg, 1990).

In the late 1980s unusually severe group-A streptococcal infections were reported from the United States and several European countries [for references see Musser et al. (1993)]. The M1 serotype was frequently associated with these hyperacute and often lethal infections. Protein H has affinity for the constant (Fc) region of IgG and the molecule is expressed by a strain (AP1) of the M1 serotype (Åkesson et al., 1990), which is also virulent to mice (Björck et al., 1989). M proteins are known to bind fibrinogen (Kantor, 1965), and previous work has demonstrated that M1 protein from the AP1 strain also has affinity for albumin and polyclonal IgG (Schmidt and Wadström, 1990). In the present work we have continued the studies of M1 protein and Protein H in the AP1 strain. The localization and structure of the M1 gene, the protein-binding properties of the M1 protein, and the relationship between M1 protein and Protein H have been analysed. The data further define some highly specific protein–protein interactions, and the results provide information which should facilitate future investigations of the molecular mechanisms underlying virulence and pathogenicity in the AP1 strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, culture media and antisera

S. pyogenes strain AP1 is strain 40/58 of the M1 serotype from the WHO Streptococcal Reference Laboratory in Prague. The strain was stored at -70°C in Todd–Hewitt broth supplemented with 20% (v/v) calf serum. For isolation of proteins, AP1 bacteria were grown in a 7-litre fermenter under the following

conditions: 5 litres of Todd-Hewitt broth was inoculated with 0.1 vol. of an AP1 overnight culture and then grown for 18 h at 37 °C. pH was adjusted to 7.2 and the glucose concentration was regulated to 0.2% (w/v). The *E. coli* strains JM109 and C600 were used as hosts for recombinant plasmids and for propagating bacteriophage λ . Bacteriophage λ EMBL3 (Frischauf et al., 1983) and plasmid vector pHD389 (Dalbøge et al., 1989) were used for cloning. Anti-M1 sera were raised in rabbits by immunizing the animals with AP1 bacteria. The antisera were adsorbed with M type 12 and M type 25 group-A streptococci.

Proteins and labelling of proteins

Polyclonal human IgG (Kabi, Stockholm, Sweden) was purified on Sephadex G-200 and Fab fragments were prepared by papain digestion of this material. IgGfC fragments were from Cappel (West Chester, PA, U.S.A.). The myeloma human IgGs used have been described previously (Myhre and Kronvall, 1980; Stenberg et al., 1992). Polyclonal human immunoglobulin light chains and monoclonal IgD was kindly provided by Dr. A. Grubb, Department of Clinical Chemistry, University of Lund, Sweden. Polyclonal human IgM and serum IgA were from Cappel. Monoclonal human IgE was a gift from Dr. I. Olsson, Department of Medicine, University of Lund, Sweden. Purified IgG antibodies from various mammalian species have been described (Kihlberg et al., 1992; de Chateau et al., 1993). Human serum albumin and fibrinogen were from Kabi and fragment D of fibrinogen was prepared as described earlier (Schmidt et al., 1984). β_2 -Microglobulin was purified from human urine (Berggård and Bearn, 1968). Purified papain-solubilized major histocompatibility complex (MHC) class-I and detergent (Nonidet P40)-solubilized MHC class-II antigens were kindly provided by Dr. L. Rask, Department of Cell Research, University of Uppsala, Sweden and Dr. J. Sundelin, Department of Medical Cell Research, University of Lund, Sweden. Recombinant Protein H was purified as previously described (Åkesson et al., 1990). Proteins were labelled by conjugation with activated horseradish peroxidase (Sigma, St. Louis, MO, U.S.A.) using the periodate oxidation method (Tijssen, 1985) under conditions described previously (Schmidt and Wadström, 1990). Proteins were radio-labelled with ^{125}I to a specific radioactivity of 2–10 $\mu\text{Ci}/\mu\text{g}$ of protein using the Bolton and Hunter (1973) reagent (Amersham, Amersham, Bucks., U.K.). Protein-bound iodine was separated from free iodine by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Plasma absorption experiments

AP1 bacteria or Protein H-Sepharose were incubated with human plasma. Overnight cultures of AP1 bacteria were washed twice with PBS (0.12 M NaCl, 0.03 M phosphate, pH 7.4) and the bacterial concentration was adjusted to 10% (2×10^{10} cells/ml). Protein H was coupled to CNBr-activated Sepharose 4B (Pharmacia) with a final concentration of 0.34 mg of Protein H/ml of Sepharose. Bacterial cells (2×10^9) were incubated with 100 μl of plasma diluted 1:8 in PBS, and 0.35 ml of Protein H-Sepharose was incubated with 100 μl of undiluted plasma. After 1 h at room temperature the bacterial cells and the Protein H-Sepharose were washed five times with PBS. Bound proteins were eluted from the cells with 100 μl of 0.1 M glycine/HCl, pH 2.0, and from Protein H-Sepharose with 200 μl of the same buffer. The pH of the eluted material was adjusted to 7.5 with 1 M Tris, and the eluted proteins were analysed by SDS/PAGE.

Purification of streptococcal and *E. coli*-expressed M1 protein

Proteins in the culture supernatant of AP1 were precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 70% saturation. The precipitate was dialysed in the presence of 0.5 mM phenylmethanesulphonyl fluoride against water and finally against 0.05 M Tris/0.15 M NaCl, pH 7.4. This crude preparation was purified on fibrinogen-Sepharose and fractions positive for peroxidase-labelled fibrinogen and albumin in slot binding experiments were purified further on albumin-Sepharose (Schmidt and Wadström, 1990).

A phage λ recombinant (λ 1:4) was used to produce lysates in liquid cultures using *E. coli* strain C600 as the propagating strain. The *E. coli* clones expressing *emm1* products in pHD389 were grown in Luria-Bertani broth containing ampicillin (100 $\mu\text{g}/\text{ml}$) at 30 °C to a D_{600} of 0.6. Protein synthesis was induced by raising the temperature to 42 °C for 4 h. Cells were harvested by centrifugation and lysed by osmotic shock (Sambrook et al., 1989). *E. coli* lysates were subjected to affinity chromatography on columns of fibrinogen, IgG or albumin, coupled to Sepharose CL-4B (Pharmacia). Columns were washed with PBS, bound peptides were eluted with 3 M KSCN, and the eluted material was dialysed against PBS.

PCR

PCRs were performed in a thermocycler (Hybaid, Teddington, Middx., U.K.), using heat-stable DNA polymerase (Promega, Madison, WI, U.S.A.) as described previously (Saiki et al., 1988). The oligonucleotides A7 (5'-GTTGCTCAGGCGGCGC-CGCTTATGAGAAAATTAACAGGTACGGCATCA-3'), A21 (5'-CGCTCCGAATTCCATGGTGACCTCTCCTTA-ACCTCATT-3'), ARA (5'-CAGCAGGCGCCGATCGAGG-GTAGGAACGGTGATGGTAATCCTAGGGAAG-3'), GBS (5'-CAGTCTAGATTATTACTCAAGTTTGTCTTTTCGATCGTTAGC-3'), AR1 (5'-CAGCAGCCATGGCGCCGATG-ACGATTACTAGAGAACAAGAGATTAAATCG-3') and GB2 (5'-CAGTCTAGATTATTATTCAAGAGCAGCTAATTTG-CTGTTTGC-3') were used as primers with genomic AP1 DNA prepared as described (Sjöbring et al., 1989). Amplifications were performed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 72 °C at 2 min. PCR products used in cloning experiments were purified using Magic PCR Preps (Promega).

Cloning procedures

The construction of a genomic library of *Sau3AI* fragments of AP1 DNA in λ EMBL3, and the screening of the library with anti-M1 serum, has been described (Åkesson et al., 1990). The M1 gene was amplified in a PCR experiment using oligonucleotides based on a consensus sequence from M protein signal sequences (A7) and on the region upstream of the Protein H gene (A21). The resulting fragment of 1.5 kb was digested with *NarI* and *EcoRI*, the recognition sites which had been introduced through the primers, and ligated to pHD389 which had been digested with the same restriction enzymes. Fragments inserted in the *NarI* site of this vector will be translated fused to the signal sequence of *E. coli* outer-membrane protein A (ompA), and are under transcriptional control of the p_R promoter. The gene encoding the temperature-sensitive λ repressor cI857 which regulates p_R has also been introduced into pHD389. Ligation mixtures were transformed to *E. coli* JM109 and transformants were grown on Luria-Bertani agar containing ampicillin (100 $\mu\text{g}/\text{ml}$) at 30 °C. For screening of relevant clones, colonies were incubated at 42 °C for 4 h, transferred to nitrocellulose filters and lysed. The filters were subsequently probed with anti-M1 sera. Standard procedures for ligation, transformation and

colony blotting were used (Sambrook et al., 1989). Restriction enzymes were from Promega.

Generation of M1 protein fragments

The region of the M1 gene corresponding to amino acids 42–232 (the A, B and S regions) was amplified using PCR with the primers ARA and GBS. Similarly, the region corresponding to amino acids 195–358 (the S and C regions, and the first 11 amino acids of the D region) was amplified with the primers AR1 and GB2. The resulting fragments of approx. 600 bp and 500 bp respectively were cloned into the vector pHD389 and screened for reactivity with anti-M1 sera as described above. Two positive clones, pAS (containing the A, B and S regions) and pSC3 (containing the S and C regions and a part of the D region), were selected and the identities of the inserts with the *emm1* sequence were controlled by DNA-sequence analysis. The clones were grown at 30 °C to mid-exponential phase and expression of the fragments was induced by increasing the temperature to 42 °C for 4 h. Cells were harvested and lysed, the supernatant of pAS was fractionated on fibrinogen–Sephacrose and the supernatant of pSC3 on albumin–Sephacrose. The eluted products were called fragment A-S and fragment S-C3 respectively.

Electrophoresis

SDS/PAGE was performed as described by Neville (1971), using a total polyacrylamide concentration of 10% and 3.3% cross-linking. Samples were boiled for 3 min in a buffer containing 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Molecular-mass markers were from Sigma. Gels were stained with Coomassie Blue. Agarose-gel electrophoresis of plasma proteins was performed as described by Johansson (1972).

Western-blot analysis and analysis of proteins directly applied to poly(vinylidene difluoride) (PVDF) membranes

After SDS/PAGE, proteins were transferred to PVDF membranes (Immobilon, Millipore, Bedford, MA, U.S.A.) by electroblotting from gels as described by Towbin et al. (1979), using a Multiphor II semi-dry blotting apparatus (Pharmacia). Transfer of proteins from agarose gels was carried out by applying PVDF membranes on top of the gels under pressure (2 kg). Proteins were also directly applied to PVDF membranes using a slot-blot apparatus from Schleicher and Schuell (Dassel, Germany). The membranes were blocked in 0.15 M veronal-buffered saline, pH 7.4, containing 0.25% gelatin and 0.25% Tween 20, then incubated with ¹²⁵I-labelled proteins, and washed as previously described (Åkesson et al., 1990). Autoradiography was performed at –70 °C using Kodak X-Omat AR films and Kodak X-Omat regular intensifying screens.

Competitive binding assays and calculation of affinity constants

The wells of microtitre plates (Nunc, Denmark) were coated with 200 µl of IgG or fibrinogen (10 µg/ml). After blocking with PBS containing 1% skimmed milk and 0.02% Tween 20, 100 µl of protein sample was added in a dilution series with an initial concentration of 1 mg/ml. To each well, 50 µl of peroxidase-labelled M1 protein (25 ng/ml) was added. After incubation for 3 h in room temperature, the wells were washed with PBS containing 0.02% Tween 20. The reaction was visualized with *o*-phenylenediamine and evaluated in a microtitre plate reader (Carl-Zeiss, Jena, Germany). Competitive binding experiments were also performed with albumin, fibrinogen, IgG or IgGFC,

coupled to polyacrylamide beads (Immunobeads, Bio-Rad, Richmond, CA, U.S.A.). Details of the procedure and affinity calculations using the formula of Scatchard have been reported (Åkerström and Björck, 1989).

DNA sequence analysis

Double-stranded DNA sequencing was performed by using the chain-termination method (Sanger et al., 1977) with Sequenase (Pharmacia). Sequencing of λ DNA was performed using the *fmol* DNA Sequencing System (Promega) according to the manufacturer's instructions. Sequence data were analysed using the GeneWorks program (IntelliGenetics, Mountain View, CA, U.S.A.).

Amino acid sequence analysis

N-terminal amino acid sequences were determined using an Applied Biosystems 477A protein sequencer.

RESULTS

Purification and binding properties of extracellular M1 protein: comparison with Protein H

The group-A streptococcal strain AP1 (40/58) used in this work has previously been shown to express Protein H, an IgGFC-binding surface protein (Åkesson et al., 1990). In addition, an extracellular protein of this strain was reported to bind fibrinogen, polyclonal IgG and albumin. The molecule was serologically related to M1 protein (Schmidt and Wadström, 1990). To characterize this extracellular protein further, proteins in AP1 culture supernatants were precipitated and purified by affinity chromatography on fibrinogen- and albumin–Sephacrose. The yield was 2–3 mg of protein from a 5-litre fermentation. The purified material was analysed by SDS/PAGE and was found to be size heterogeneous with two bands of 49 and 47 kDa (Figure 1, lane 1). The N-terminal amino acid sequence of this material was determined to be Asn-Gly-Asp-Gly-Asn-Pro-Arg-Glu-Val-Ile, a sequence that was identical with the M1 sequences previously published (Kraus et al., 1987; Haanes-Fritz et al., 1988; Harbaugh et al., 1993). The N-terminal sequence identity with M1 protein, the ability to bind fibrinogen and the fact that the fibrinogen-binding protein of the AP1 strain has both M1

Figure 1 SDS/PAGE and Western-blot analysis of M1 protein and Protein H

Proteins were separated by SDS/PAGE (10% gels) under reducing conditions. After electrophoresis one gel was stained with Coomassie Blue (a). Lane 1, M1 protein purified from streptococcal growth medium; lane 2, material from λ1:4 phage lysate purified on fibrinogen–Sephacrose; lane 3, material from λ1:4 phage lysate purified on IgG–Sephacrose; lane 4, recombinant Protein H purified on IgG–Sephacrose. Two additional and identical gels were blotted on to PVDF filters and probed with [¹²⁵I]IgGFC (b) or [¹²⁵I]fibrinogen (c).

Figure 2 Slot binding analysis showing the binding specificity of (a) M1 protein and (b) Protein H

Various amounts of the IgG subclasses, fragments of IgG, immunoglobulins of other classes, β_2 -microglobulin, MHC class I and class II, human serum albumin and fibrinogen were applied to PVDF filters. The filters were incubated with radiolabelled M1 protein or Protein H (2×10^5 c.p.m./ml for 3 h, autoradiographed for 3 days).

Figure 3 SDS/PAGE analysis of human plasma proteins eluted from AP1 streptococci or from Protein H-Sephadex

Proteins were separated by SDS/PAGE (10% gels) under reducing conditions. After electrophoresis the gel was stained with Coomassie Blue. Lane A, human plasma diluted 1:50; lane B, proteins eluted from AP1 streptococci at pH 2.0; lane C, proteins eluted from Protein H-Sephadex at pH 2.0; lane D, human albumin (3 μ g); lane E, human fibrinogen (5 μ g); lane F, human IgG (5 μ g).

protein sequence similarity and been shown to adsorb antibodies with bactericidal activity from M1-specific antisera (Kühnemund et al., 1985) confirmed that the extracellular protein was M1 protein.

The protein-binding properties of extracellular M1 protein and Protein H were then analysed and compared. Figure 2 shows binding of the two molecules, radiolabelled with 125 I, to various human proteins immobilized on PVDF filters. Both radiolabelled proteins showed affinity for polyclonal IgG, myeloma IgG of all four subclasses and IgGfC fragments, whereas no binding was seen to IgGfAb. These results map IgG-binding to the Fc region. Stronger signals were obtained with Protein H, which was explained when the affinity was determined for the reaction between polyclonal IgG and M1 protein. Thus the affinity constant was about 500-fold lower than for Protein H (see below). Neither protein showed affinity for other classes of

Figure 4 Binding of M1 protein (a) and Protein H (b) to IgG from different mammalian species

Dilution series of polyclonal IgG from different species were applied to PVDF filters in a slot-blot apparatus. The filters were probed with radiolabelled M1 or Protein H as described in the legend to Figure 2.

immunoglobulin or other members of the immunoglobulin super family, except that Protein H bound to MHC class-II antigens. This observation, which is currently being analysed in more detail, may indicate that Protein H can act as a super antigen. As reported, M1 protein had affinity for albumin and fibrinogen (Schmidt and Wadström, 1990), whereas Protein H reacted with albumin but not with fibrinogen. The binding of Protein H to albumin was not detected in earlier experiments (Åkesson et al., 1990), probably because of radiolabelling of Protein H by a different procedure.

To demonstrate the binding properties of intact AP1 streptococci, a bacterial suspension was incubated with human plasma. After extensive washing, proteins bound to the bacterial cells were eluted with glycine buffer, pH 2.0, and analysed by SDS/PAGE (Figure 3, lane B). Several protein bands were obtained and the most prominent were identified in immunoblots (not

Figure 5 Binding of M1 protein to monoclonal human IgG, albumin and fibrinogen separated by agarose-gel electrophoresis

A portion (2 μ l) of each sample was run on 1% (w/v) agarose gels in veronal buffer. One gel was stained (a) and one was blotted on to a PVDF membrane and probed with 125 I-labelled M1 protein (b) as described in the legend to Figure 2. Two different myeloma IgGs of each subclass were applied at a concentration of 1 mg/ml: IgG1 (lanes 1 and 2), IgG2 (lanes 3 and 4), IgG3 (lanes 5 and 6) and IgG4 (lanes 7 and 8). Lane 9, human serum albumin (1 mg/ml); lane 10, fibrinogen (1 mg/ml). Human plasma and serum were diluted eight times in veronal-buffered saline, pH 8.6, and applied to the indicated lanes.

shown) as albumin (66 kDa), IgG heavy (50 kDa) and light (25 kDa) chains and fibrinogen (three bands between 47 and 64 kDa). A similar experiment in which Protein H coupled to Sepharose was incubated with human plasma showed that Protein H adsorbed albumin and IgG (Figure 3, lane C). These results show that AP1 bacteria have the ability to bind several human plasma proteins and further confirm the albumin-binding property of Protein H (Frick et al., 1994).

The interaction between IgG and M1 protein was further analysed. In Figure 4 IgG from various mammalian species was tested for binding. Apart from human IgG, baboon, rabbit, guinea-pig and pig IgG also reacted with M1 protein, whereas four other species (rat, mouse, cow, and horse) were negative. Protein H did not interact with guinea-pig IgG but otherwise showed the same binding pattern. In Figure 5(a) eight human IgG myeloma proteins (two of each subclass), albumin, fibrinogen, human plasma and serum were separated by agarose-gel electrophoresis. The proteins were transferred to PVDF membranes and probed with 125 I-labelled extracellular M1 protein (Figure 5b). All myeloma proteins, albumin and fibrinogen reacted with the probe, whereas in plasma the γ -region and the bands corresponding to fibrinogen and albumin bound M1 protein. Under these experimental conditions, no other M1-protein-binding activity was seen among the separated plasma proteins. The serum sample confirmed the specificity of the interaction between fibrinogen and M1 protein. When Protein H was used as the probe in identical experiments, all myeloma proteins, albumin, as well as the serum and plasma bands

corresponding to the γ -region and albumin, reacted with the probe (results not shown).

Cloning and expression of the *emm1* gene of the AP1 strain in *E. coli*

A library of AP1 DNA was constructed in λ EMBL3 and recombinant plaques were screened with anti-M1 antibodies. Positive plaques were picked, purified and subjected to another three rounds of screening. One positive λ clone (λ 1:4) was selected for further study. A phage lysate of λ 1:4 was subjected to affinity chromatography on fibrinogen-Sepharose. Material eluted from the affinity column was size heterogeneous with a major band of 55 kDa which reacted also with [125 I]IgGfc in Western-blot experiments (Figure 1, lane 2). The higher molecular mass of recombinant M1 compared with the M1 protein purified from streptococcal growth medium could be explained by C-terminal cleavage of M1 protein before release into the medium (Pancholi and Fischetti, 1989).

In the work of Gomi et al. (1990), the Protein H gene (*sph*) was sequenced. A sequence was also determined directly upstream of *sph* which could represent the 3' end of a possible M-protein gene. To test this hypothesis, a λ 1:4 phage lysate was subjected to affinity chromatography on IgG-Sepharose to analyse whether Protein H was present in the lysate. The eluted protein was compared with Protein H on SDS/PAGE and in a Western blot (Figure 1, lanes 3 and 4). Both proteins had major bands of approx. 42 kDa. Protein H has a tendency to form dimers (Åkerström et al., 1992), which explains the additional bands of higher molecular mass. Radiolabelled IgGfc, but not fibrinogen, reacted with the two proteins in these experiments indicating that λ 1:4 contained the genes for both M1 protein and Protein H. The λ 1:4 lysate purified on IgG-Sepharose also showed a faint band of about 55 kDa on SDS/PAGE corresponding to the M1 protein. Radiolabelled fibrinogen gave a weak positive reaction with this band when the autoradiograph was exposed for several days (not seen in Figure 1). The low yield of the M1 protein in this experiment was probably due to the 500-fold lower affinity of IgG compared with that of Protein H. The expression level of *emm1* in λ 1:4 was low. Thus a PCR fragment of the gene was amplified and cloned into the inducible vector pHD389 (see the Materials and methods section). M1-protein-expressing clones were used for the production of M1 protein (20–30 mg/l of stationary culture), and this material, purified by affinity chromatography on albumin- and fibrinogen-Sepharose, represents the M1 protein in subsequent experiments. The affinity constants for the reactions between M1 protein and albumin, fibrinogen and IgG were determined by Scatchard plots. Figure 6 summarizes the results and also gives the affinities for the reactions between Protein H and the three plasma proteins. The M1 protein-albumin plot appears slightly concave which could indicate more than one binding site. However, as the correlation coefficient was -0.97 , an affinity constant calculation was still performed.

Sequencing of the *emm1* gene

The nucleotide sequence of AP1 *emm1* was determined from two independent PCR clones using synthetic primers derived from known sequences. The sequence was confirmed and extended by performing sequencing reactions on λ 1:4 phage DNA using specific primers. A region of 1666 bp was sequenced and found to contain an open reading frame extending from position 162 to 1614 encoding a protein of 484 amino acid residues. The first 41 predicted residues were similar to signal peptides of other M1

Figure 6 Scatchard plots for the reactions between ¹²⁵I-labelled M1 protein and immobilized IgG, albumin and fibrinogen

A constant amount of [¹²⁵I]M1 protein was mixed with constant amounts of IgG- (a), albumin- (b) or fibrinogen- (c) coupled polyacrylamide beads followed by the addition of unlabelled M1 protein in different concentrations. After 16 h at 20 °C the beads were washed and centrifuged, and the radioactivity in the pellets was determined. Bound and free M1 protein concentrations were calculated and plotted. The equilibrium constants for the reactions in (a), (b) and (c) were 3.4×10^6 , 5.7×10^9 and $1.1 \times 10^{10} \text{ M}^{-1}$ respectively, and those for the reactions between Protein H and IgG (Åkesson et al., 1990) and Protein H and albumin (Frick et al., 1994) are 1.6×10^9 and 7.8×10^9 respectively; there was no binding between Protein H and fibrinogen.

and M-like proteins. Also, the N-terminal amino acid sequence of the purified M1 protein aligned with residues 42–51 of the deduced amino acid sequence, suggesting that the signal sequence was cleaved between residues 41 (Ala) and 42 (Asn). Thus the mature M1 protein was expected to be 443 residues long with a calculated molecular mass of 50.2 kDa, a value that corresponds well to an apparent molecular mass of 55 kDa on SDS/PAGE, as molecular masses of M proteins determined by SDS/PAGE are known to be overestimated (Hollingshead et al., 1986). Upstream from the ATG start codon, there were sequences matching the consensus sequences for Gram-positive bacterial promoters and ribosomal-binding sites. The last 158 nucleotides of the coding sequence and all of the downstream flanking sequence obtained from the sequencing of *emm1* were identical with the sequence upstream of *sph*. Thus it was shown that the genes encoding M1 protein and Protein H are linked on the chromosome with a distance of 214 bp.

Like the other proteins in the M protein family, M1 consisted of distinct repeat regions (Figures 7 and 8). The mature protein had a unique sequence of 91 amino acid residues at the N-terminus. This region was followed by two repeated sequences of 28 residues each (B1, amino acid residues 133–160; B2, amino acid residues 161–188) with 82% internal similarity. Directly after the end of B2 there was a six-amino acid residue stretch identical with the beginning of B1 and B2. Following the B repeats a non-repeated sequence of 35 residues (S) was identified, followed by another repeat region consisting of three units of 42, 42 and 31 residues respectively (C1, amino acid residues 233–274;

Figure 7 Comparison of the AP1 M1 protein sequence with previously reported M1 protein sequences (M1.0, M1.1, M1.2)

The deduced amino acid sequence of the AP1 M1 protein is shown. Arrows mark the beginning of the various domains. The start of the A domain represents the N-terminus of the mature protein. Non-identical amino acid residues in the compared sequences are indicated.

Figure 8 Amino acid sequence comparison between M1 protein and Protein H

Numbers below the outlined sequences correspond to the first amino acid residue of the different regions. The percentage identity between the regions in the proteins indicated between the sequences were calculated by dividing matched residues by the number of residues in the shortest sequence. Fragments A-S and S-C3 of M1 protein are indicated by the bars.

C2, amino acid residues 275–316; C3, amino acid residues 317–347). The similarity of these three repeats was 94–95%. The rest of the molecule consisted of the D region, including the putative wall-spanning and membrane-anchoring domains (Fischetti, 1989).

Comparison of the AP1 *emm1* sequence with other M1 protein sequences

Two complete and one partial M1 gene sequence from different strains of the M1 serotype were recently reported (Harbaugh et

Figure 9 Competitive binding experiments

The binding of peroxidase-labelled M1 to fibrinogen (a) or IgG (b) immobilized on microtitre plates was inhibited with different amounts of fibrinogen (○), fragment D of fibrinogen (●), serum albumin (✱) or IgG (△).

al., 1993). The *emm1* from AP1 was identical with the partly sequenced *emm1.0* except for one nucleotide shift which did not affect the deduced amino acid sequence (C instead of T in position 1200 in the coding sequence). Of the complete sequences, the AP1 *emm1* was most similar to *emm1.2* differing in eight base pairs causing a change in six amino acid residues. The second complete sequence, *emm1.1*, had 37 nucleotide substitutions compared with AP1 *emm1*, causing 20 amino acid substitutions. The deduced amino acid sequence of M1 protein (AP1) and a comparison with the sequences of the previously sequenced M1 proteins is shown in Figure 7.

Sequence comparison with M proteins of other serotypes and M-like immunoglobulin-binding proteins

The amino acid sequence of M1 was also compared with the other M proteins and M-like immunoglobulin-binding proteins sequenced to date (Hollingshead et al., 1986; Robbins et al., 1987; Miller et al., 1988; Mouw et al., 1988; Haanes and Cleary, 1989; Heath and Cleary, 1989; Frithz et al., 1989; Manjula et al., 1991; O'Toole et al., 1992; Bessen and Fischetti, 1992), including Protein H (Gomi et al., 1990). The signal sequence and the D region showed similarity to the corresponding regions in all these proteins, varying from 71 to 83% in the signal sequence, and from 67 to 100% in the D region. The large majority of the sequenced M proteins and M-like proteins have C repeats. C repeats of eight of these proteins were compared with the C repeats of M1 protein from strain AP1. The similarity varied between 50 and 100% (mean value 75.8%), whereas no significant similarity (less than 30%) was found in the N-terminal regions of the various proteins. Figure 8 shows the similarity between M1 protein and Protein H of the AP1 strain. The comparison is representative for the structural relation between M proteins and immunoglobulin-binding M-like proteins having C repeats.

Mapping of protein-binding sites on M1 protein

The relationship between the interactions of IgG, fibrinogen and albumin with M1 protein was studied in competitive binding experiments. Figure 9 shows that the binding of peroxidase-

labelled M1 protein to immobilized fibrinogen was inhibited by unlabelled fibrinogen, fragment D of fibrinogen and IgG. Albumin did not affect the binding. The binding of M1 to immobilized IgG was also inhibited by fibrinogen and IgG but not by fragment D of fibrinogen or albumin. Thus fibrinogen and IgG compete for binding to M1 protein, whereas the smaller fragment D of fibrinogen only inhibited the binding of intact fibrinogen. The data indicate separate binding sites on M1 protein for the three plasma proteins, and that the binding sites for IgG and fibrinogen are close enough to allow mutual inhibition.

In order to map further the binding sites on the M1 molecule, two fragments of the *emm1* gene were expressed in *E. coli* (see the Materials and methods section and Figure 8). The fragment A-S was purified on fibrinogen-Sepharose and labelled with ^{125}I . This probe bound to fibrinogen and myeloma IgG1 immobilized on PVDF membranes but not to albumin (not shown). Fragment S-C3 was purified on albumin-Sepharose and also showed affinity for IgG but not for fibrinogen (Figure 10). The results map albumin binding to the C repeats, fibrinogen binding to the A-B3 region, and IgGFc binding to the S region.

Further analysis of the interaction between IgG and M1 protein

As described above, M1 protein interacts with IgG through the Fc region. Previous work has demonstrated that Protein A of *Staphylococcus aureus*, Protein G of group C and G streptococci, and FcRA76 and Protein H of *S. pyogenes* all bind to the $\text{C}\gamma 2\text{--C}\gamma 3$ interface region of IgGFc (Deisenhofer, 1981; Stone et al., 1989; Heath et al., 1990; Frick et al., 1992). Experiments were designed to determine if M1 protein also had affinity for this region. In a first set of experiments it was indeed found that intact M1 protein, as well as fragments A-S and S-C3, could inhibit the binding of ^{125}I -labelled Protein H to IgGFc immobilized on polyacrylamide beads (not shown). Moreover, binding of ^{125}I -labelled M1 protein to IgG1 on beads was inhibited by unlabelled M1 protein, fragments A-S and S-C3 and Protein H (Figure 11). The results demonstrate that M1 protein also interacts with IgGFc in the $\text{C}\gamma 2\text{--C}\gamma 3$ interface region. However, as judged from the curves of Figure 11, the affinity for IgG is more than 100-fold higher for intact M1 protein than for the two fragments. Protein H is known to form dimers that exhibit a

Figure 10 Binding properties of fragment S-C3 (b) compared with intact M1 protein (a)

Dilutions of human myeloma IgG1, serum albumin and fibrinogen were applied to PVDF filters in a slot-blot apparatus. The filters were incubated with radiolabelled M1 protein or fragment S-C3 (2×10^5 c.p.m./ml) for 3 h and autoradiographed for 3 days.

Figure 11 Inhibition of ^{125}I -labelled M1 protein binding to immobilized IgG

Different amounts of M1 protein (●), fragments A-S (▲) and S-C3 (△) of M1 protein and Protein H (○) were used as inhibitors in competitive binding experiments (left). The inhibitors subjected to SDS/PAGE (10% gels) are shown to the right.

higher affinity for IgG than the monomer (Åkerström et al., 1992). If the same is true for M1 protein, the decrease in affinity for the fragments could be explained by the inability to form dimers. Another explanation is that removal of the surrounding amino acid residues changes the conformation of the S region.

On the M1 molecule the S region was found to be responsible for IgG binding (see above). When aligning the sequence of S

Figure 12 Sequence similarity between the S region of M1 protein and the A repeat region of FcRA76

M1 protein and FcRA76 are schematically represented and the different regions of the proteins are denoted. The shaded areas are aligned and residues in FcRA76 identical with M1 protein are boxed. Numbers correspond to amino acid residue positions in the sequences.

with sequences of M proteins and M-like immunoglobulin-binding proteins, the most similar sequences were found in two proteins: FcRA76 and Mrp4. These molecules are IgG-binding surface molecules of *S. pyogenes* which, in contrast with other M-like proteins, do not have C repeats (Heath and Cleary, 1989; O'Toole et al., 1992). Instead, both proteins have two almost identical so-called A repeats, and within this repeat region two areas were found showing similarity to the S region of M1 protein (Figure 12). Interestingly, the A repeats of FcRA76 were reported to bind IgG (Heath et al., 1990). No similarity was found between the S region and the IgG binding regions of Proteins A, G or H.

DISCUSSION

Previous work has demonstrated that streptococci in the plasma environment are coated with human plasma proteins (Kronvall et al., 1979), which changes the physicochemical surface properties of the bacteria (Miörner et al., 1980). In the present study, the interactions between M1 protein, a surface protein of *S. pyogenes*, and the three most abundant human plasma proteins (albumin, IgG and fibrinogen) were analysed in more detail. The results show that M1 protein has developed highly specific and separate binding sites of these proteins.

Protein G of group C and G streptococci was originally described as an IgG binding surface protein (Reis et al., 1984; Björck and Kronvall, 1984), but later work demonstrated that the molecule could also bind albumin (Björck et al., 1987). This interaction was located to repeats in the N-terminal half of Protein G (Åkerström et al., 1987), whereas other repeats in the C-terminal part were responsible for IgG binding (Fahnestock et al., 1986; Guss et al., 1986). Here we find that M1 protein, just like Protein G and Protein H, has separate IgG binding- and albumin-binding regions. Moreover, as in Protein H (Frick et al., 1994), albumin-binding is located to the C repeats. As the C repeats are quite similar in various M proteins and M-related immunoglobulin-binding proteins this was not unexpected. The results suggest that other proteins with C repeats will also exhibit albumin-binding activity.

The interaction between M proteins and fibrinogen was originally described by Kantor (1965). Fibrinogen binding to M1 protein was mapped to the N-terminal part of the molecule, a part that shows a low degree of sequence similarity to other M proteins. Nevertheless, when 49 strains of *S. pyogenes* of different M serotype were tested for fibrinogen binding, 45 were clearly positive (H. BenNasr and L. Björck, unpublished work), supporting the notion that the three-dimensional structure of this

region is similar in various M proteins despite sequence heterogeneity (Fischetti, 1989). The fact that Protein H does not bind fibrinogen may therefore indicate a different structural organization of this region in Protein H. It has been reported that fibrinogen binding to M protein has an inhibitory effect on complement-mediated opsonization and phagocytosis of *S. pyogenes* (Whitnack and Beachey, 1985), but the exact role of fibrinogen in streptococcal clearance is still not fully understood. In this context it should be mentioned that M proteins have also been demonstrated to bind factor H of the complement system (Horstmann et al., 1988) which could help to explain the antiphagocytic property of M protein.

The M1 protein of the AP1 strain was found to bind IgGfC. However, it is most likely that M1 proteins of other strains will also bind IgGfC as the S region responsible for the interaction is highly conserved (Figure 7). *S. pyogenes* causes a variety of serious infections. This century has seen a decline in rates and severity of these infections, but in the 1980s this tendency was interrupted by a world-wide resurgence in serious systemic infections particularly associated with streptococci of the M1 serotype (Musser et al., 1993; Martin and Single, 1993). Whether the IgGfC-binding activity of M1 protein contributes to the pathogenicity and/or virulence of M1 strains can now be studied in the AP1 strain. This strain is virulent to mice and we are at the moment, by allelic replacement, trying to create AP1 mutants in which the S region is deleted. The virulence of these mutants will then be tested in the mouse model. Another question concerns how the simultaneous expression of two IgGfC-binding proteins, M1 protein and Protein H, will affect virulence. Mutants missing *emm1* and/or *sph* are therefore being generated by transposon-mediated mutagenesis by the methods of Caparon and Scott (1991). We are also currently analysing a number of M1 strains isolated from Swedish patients with toxic, and in some cases lethal, infections (Holm et al., 1992), to determine the presence, localization and structure of the *emm1* and *sph* genes in these strains. The molecular data presented here are essential to these studies focusing on the biological behaviour of M1 protein and Protein H.

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