A Protein G-Related Cell Surface Protein in *Streptococcus zooepidemicus*

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This work describes the cloning and sequencing of a gene encoding a plasma protein receptor from *Streptococcus zooepidemicus*. This receptor, termed protein ZAG, is a 45-kDa protein that binds α_2-macroglobulin (α_2M), serum albumin, and immunoglobulin G (IgG). The IgG-binding activity is located in the C-terminal part of the molecule and is mediated by two repeated domains highly homologous to each other as well as to the corresponding domains in streptococcal type III Fc receptors. The IgG-binding profile of protein ZAG is similar to that previously reported for *S. zooepidemicus*. Binding to serum albumin is mediated by a short amino acid sequence in the middle of the molecule. This domain shows homology to previously described albumin-binding proteins from streptococci, and the albumin-binding profile of protein ZAG is similar to that of streptococcal protein G. The N-terminal part of protein ZAG, which mediates binding to the plasma proteinase inhibitor α_2M, is composed of a unique stretch of amino acids. Protein ZAG competes for the same, or nearby, binding site(s) in α_2M as do two recently described *Streptococcus dysgalactiae* receptors, although the sequences of the α_2M-binding domains in these three receptors show only minor sequence similarities.

The serological group C streptococci *Streptococcus zooepidemicus* and *Streptococcus equi* are the most commonly occurring etiological agents in a variety of diseases in horses (28). The former species occurs as an opportunistic commensal on the mucosae of horses and other animals and is also occasionally involved in diseases in humans (7).

Like other streptococcal species, *S. zooepidemicus* specifically binds, through cell surface components, a number of host-derived proteins such as immunoglobulin G (IgG) (18), serum albumin (31), fibronectin, collagen (16), and α_2-macroglobulin (α_2M) (17). These host-parasite recognition components might be of importance as virulence factors by acting as adhesins, antiopsonins, or host mimicry factors.

The ability to bind IgG from various species and of different subclasses has led to the classification of bacterial IgG-binding proteins in six different Fc receptor types (for a review, see reference 5). According to this classification, the IgG receptors from *S. zooepidemicus* are distinct from the well-studied type I and type III Fc receptors, staphylococcal protein A and streptococcal protein G, respectively.

We have recently cloned and sequenced two genes encoding IgG-binding proteins from two *Streptococcus dysgalactiae* strains isolated from cases of bovine mastitis (13, 14). These proteins, called protein MIG and protein MAG, were highly homologous in their IgG-binding domains as well as to protein G from group G streptococci. Protein MIG contained five IgG-binding domains, while protein MAG only contained one such domain. Protein MAG also bound serum albumin by means of a 50-amino-acid-long region located in the middle of the molecule, which was partially homologous to the albumin-binding domain of protein G. Interestingly, both protein MIG and protein MAG also bound the proteinase-complexed form of the plasma proteinase inhibitor α_2M, the so-called fast form

of α_2M (2). This binding was in both cases mediated by an N-terminally located unique amino acid sequence.

In this work, we report on the cloning, sequencing, and expression of an IgG-binding protein from *S. zooepidemicus* Z5, called protein ZAG. This protein shows functional similarities to streptococcal type III Fc receptors. Protein ZAG is similar in architecture and function to the two *S. dysgalactiae* receptors MIG and MAG, combining binding of IgG and albumin with the binding of the plasma proteinase inhibitor α_2M. Interestingly, protein ZAG represents a third variant of α_2M-binding capability that we have found in streptococci.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. zooepidemicus* Z5 was obtained from Eva Olsson, The Swedish National Veterinary Institute, Uppsala, Sweden. *Escherichia coli* DH5α [F$^-$ ϕ80d *lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*] was used as the host strain in cloning and expression of recombinant proteins. The streptococcal strain was grown on blood agar plates or in Todd-Hewitt broth (Difco) at 37°C. *E. coli* was grown in Luria-Bertani (LB) medium. In cases of antibiotic selection, ampicillin was added to the *E. coli* growth medium to a concentration of 50 μg/ml.

Proteins and reagents. IgG-Sepharose 6FF was obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden. Serum albumins and IgGs from human, dog, horse, pig, rabbit, cat, cow, guinea pig, rat, sheep, goat, mouse, and hen ovalbumin were obtained from Sigma, St. Louis, Mo. Bovine serum albumin (fraction V, radioimmunoassay grade) was obtained from U.S. Biochemical, Cleveland, Ohio. Nitrocellulose (NC) filters (obtained from Schleicher & Schüll, Dassel, Germany) were used to bind proteins in dot blots or Western blots (immunoblots). The molecular weight marker kit used in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was obtained from Bio-Rad, Richmond, Calif. Horseradish peroxidase (HRP)-conjugated bovine α_2M and human serum albumin (HSA) were a gift from K. Jacobsson. HRP-conjugated rabbit IgG used in Western blotting was from Sigma; a goat anti-rabbit IgG–HRP conjugate (Bio-Rad) was used in the dot blot assay to investigate the albumin-binding profile of protein ZAG.

Preparation of DNA. Streptococcal cells were grown overnight in Todd-Hewitt broth supplemented with 0.6% yeast extract and 10 mM glycine. The next morning, glycine was added to a concentration of 0.67 M, and incubation was continued for 2 h. After harvest, the cells were washed three times in 50 mM Tris-HCl (pH 7.0) and resuspended to 1/20 of the original culture volume in the same buffer including 25% sucrose. Lysozyme was added to a final concentration of 30 mg/ml, and the suspension was incubated with gentle agitation for 2 h at 37°C. The cells now converted to protoplasts were pelleted and resuspended in 50 mM Tris-HCl (pH 7.0)–50 mM EDTA including 1% SDS and incubated at 65°C for 15 min. Cell debris was removed by centrifugation, and the

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viscous supernatant was further treated as described elsewhere for chromosomal DNA preparations (24). Phage DNA and plasmid DNA were prepared by standard methods (24).

Construction and screening of a genomic library from *S. zooepidemicus* Z5. A number of *S. zooepidemicus* strains were screened for binding of ^{125}I labelled IgG. Among the IgG-binding strains one, called Z5, was chosen. Chromosomal DNA from strain Z5 was partially digested with *Sau*3A, and fragments ranging from 7 to 20 kb were ligated to λ EMBL3 arms (Stratagene, La Jolla, Calif.) and packed in vitro, using a Packagene in vitro package kit (Promega, Madison, Wis.). The resulting library was screened for clones expressing IgG-binding activity, using ^{125}I-labelled IgG as described previously (10).

Isolation of positive subclones. Among the positive clones isolated in the screening of the λ library by using iodinated IgG, one, called λSZG1, was chosen for further studies. Subclones from λSZG1 were generated by *Eco*RI digestion and subsequent ligation to pUC18. Screening of subclones expressing IgG-binding activity was performed as described recently (14). One clone, designated pSZG40, was chosen for further studies.

DNA sequencing and analysis of the sequence. The insert in clone pSZG40 was sequenced by the dideoxy method, using Sequenase version 2.0 (U.S. Biochemical). The sequencing samples were analyzed on wedge-shaped 6% acrylamide gels containing 7 M urea. To obtain the DNA sequences of both strands of the *zag* gene, the sequencing strategy included the generation of subclones suitable for double-stranded sequencing by using commercial primers. In appropriate cases, specific synthetic oligonucleotides were used. The PC Gene DNA and protein data handling package (Intelligenetics, Inc.) was used for analysis of the DNA and protein sequences.

Expression and purification of protein ZAG and ZAG-derived peptides. The protein product encoded by plasmid clone pSZG40 was purified by affinity chromatography on IgG-Sepharose. *E. coli* cells harboring pSZG40 were grown in LB medium. The cells were pelleted, washed once with phosphate-buffered saline (PBS), and resuspended in lysis buffer (50 mM Tris-HCl [pH 7.9], 50 mM EDTA). Lysozyme was added to a final concentration of 10 mg/ml, and the suspension was incubated at 37°C for 1 h. The cell suspension was then briefly sonicated, and the cell debris was pelleted by centrifugation. The supernatant was diluted with PBS and passed over an IgG-Sepharose column. After washing, the bound protein material was eluted with 1 M acetic acid (pH 2.8). The eluted material was lyophilized and finally dissolved in distilled H_2O.

Two different gene fusion vectors were used to express various parts of the *zag* gene (Fig. 1 and 2A). The 518-bp *Xmn*I-*Hha*I fragment (positions 174 to 691) was cloned in the pMalC2 vector (New England Biolabs, Beverly, Mass.), and this clone, called pZAG2, expressing 173 amino acids from protein ZAG, was fused to maltose-binding protein (MBP). Similarly, cloning of the 508-bp *Hha*I-*Hinc*II fragment (positions 695 to 1204) generated clone pZAG3. Virtually the whole gene, including membrane- and wall-spanning domains but lacking a part of the signal sequence, was represented by clone pZAG1, which contains a 1,878-bp *Xmn*I-*Xmn*I fragment (position 174 to 2052). In this case also, the clone is a fusion with the pMalC2 vector. Finally, to construct a clone expressing the region of protein ZAG displaying homology to the albumin-binding domain of protein MAG, PCR amplification was performed with pSZG40 as the template. The following PCR primers were used: primer 1, 5′-CGGGAAGATCTT<u>CGGA CATTACAGGAGCAGCC</u>-3′ (positions 570 to 589); and primer 2, 5′-GGAA<u>T TCTGCTGCGTCAATGACCTC</u>-3′ (positions 754 to 734). The amplified product was purified, digested with *Bgl*II and *Eco*RI, and cloned into plasmid vector pGEX-2T (Pharmacia LKB Biotechnology, Uppsala, Sweden). In this construct, designated pZAG4, the PCR-amplified fragment was fused to the gene encoding glutathione *S*-transferase. Expression of each fusion protein was induced by the addition of isopropylthiogalactopyranoside (IPTG) to the growth medium.

SDS-PAGE and Western blotting. After induction, *E. coli* cells harboring the various plasmid constructs were pelleted, resuspended, and boiled for 5 min in loading buffer, containing 5% β-mercaptoethanol and 2.5% SDS, before application on three parallel gels. SDS-PAGE was performed with the Phast system (Pharmacia LKB Biotechnology), using precast 8 to 25% gradient gels and SDS buffer strips. For transfer of the separated proteins to NC filters, a filter was put on top of the gels and the temperature was raised to 55°C to increase the diffusion. After 45 min, the filters were removed and saturated in a solution of PBS containing 0.05% (vol/vol) Tween 20 (PBST) for 30 min under gentle agitation at 37°C. The filters were then incubated for 1 h in PBST supplemented individually with HRP-labelled bovine α_2M, HSA, and rabbit IgG. After washing with PBST and PBS, the bound labelled serum proteins were visualized by the addition of a 4-chloro-1-naphthol solution as a substrate for HRP.

Dot blot assay for albumin binding. Albumins of different species origin were dissolved in PBS and twofold stepwise diluted in PBS in a microtiter plate. The sample in the first well contained 10 μg of albumin, while the last well (well 8) contained 0.08 μg. After dilution, the samples were transferred to an NC filter (previously soaked in PBS) by using a dot blot apparatus (Bio-Rad). After application of the samples, the dot blot wells were washed twice with 100 μl of PBS, and the filter was removed and placed in a PBST solution. After 20 min at room temperature (RT) and with two changes of the PBST solution, the filter was placed in a PBST solution containing 3 μg of protein ZAG per ml and incubated at RT for 1 h under gentle agitation. The filter was washed with PBST and incubated for 1 h at RT with peroxidase-labelled goat anti-rabbit IgG (Bio-Rad) diluted 1:1,000 in PBST. After extensive washing with PBST and PBS, the bound IgG-HRP was detected by addition of the substrate 4-chloro-1-naphthol.

Dot blot assay for IgG binding. By using the same type of dot blot assay as described above, the IgG-binding profile of protein ZAG was studied. After application of the different IgG samples, the filter was incubated with 3 μg of protein ZAG per ml. The IgG-bound protein ZAG was detected by using an HSA-HRP conjugate.

BIAcore analysis. Surface plasmon resonance measurements were performed by using the BIAcore biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden). Protein ZAG was immobilized on sensor chip CM5, using amine coupling as described previously (12). The sensor chip was activated by injection of a solution containing 50 mM *N*-hydroxysuccinimide and 200 mM N-ethyl-*N*′-(dimethylaminopropyl)carbodiimide. Affinity-purified protein ZAG was diluted to 20 μg/ml in 10 mM sodium acetate (pH 4.5) and injected over the sensor surface for 7 min. After coupling, the excessive reactive groups on the sensor chip were deactivated by injection of 1 M ethanolamine hydrochloride (pH 8.5). With this procedure, close to 1,100 resonance units of ZAG were immobilized to the sensor surface. Before analysis of the interaction of the different IgGs, HSA, or α_2M with protein ZAG, the IgG samples were diluted to 40, 20, 10, and 5 nM with *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, 0.05% BIAcore surfactant P20). The corresponding dilutions were 1,100, 550, 275, and 137.7 nM for HSA and 200, 100, 50, and 25 nM for α_2M. The binding of each ligand to immobilized ZAG was monitored for 3 min, and the dissociation of bound ligand was monitored in buffer flow for 10 min. To dissociate bound ligand and to prepare the surface for a new injection of the ligand, the surface was regenerated with a short pulse (1 min) of 10 mM glycine-HCl (pH 1.75). The flow rate was 5 μl/min. The obtained data were analyzed by assuming a 1:1 interaction model (15) and using BIAcore evaluation software.

Inhibition assay. By using the method described by Rantamäki and Müller (22), *S. zooepidemicus* Z5 cells were treated with guanidinium chloride and coated onto flat-bottom microtiter plates (Maxisorp; Nunc, Copenhagen, Denmark). A mixture of bovine α_2M-HRP conjugate diluted 1:2,000 and increasing amounts of the lysates to be tested for inhibition was then added to the wells in duplicate. After 2 h of incubation at RT, the plate was washed several times with PBST and the bound α_2M-HRP conjugate was detected by addition of 3,3′,5,5′-tetramethyl benzidine (Boehringer Mannheim, Mannheim, Germany) as the substrate. After conversion of the blue color to yellow by addition of 1 M H_2SO_4, the plate was read at 450 nm, using a Biotek enzyme-linked immunosorbent assay reader. Prior to use, the lysates were checked by gel electrophoresis and diluted to the same approximate protein concentration. A Western blot of the lysates confirmed that the lysates from clones pMIG2 (14), pMAG2 (13), and pZAG2 bound α_2M, while the lysate from clone pZAG3 was negative in α_2M binding (data not shown).

Nucleotide sequence accession number. The *zag* sequence accession number in GenBank is U25852.

RESULTS

Sequence of the *zag* gene and deduced amino acid sequence of protein ZAG. A gene library of chromosomal DNA from *S. zooepidemicus* Z5 was screened for clones expressing IgG-binding activity. After primary isolation of a clone called λSZG1, the insert was subcloned into a plasmid vector and a clone, containing a 2.9-kb *Eco*RI fragment, which expressed IgG-binding activity was isolated. This clone was designated pSZG40. DNA sequence analysis showed that the 2.9-kb *Eco*RI fragment in pSZG40 contained an open reading frame of 1,287 nucleotides starting with an ATG codon at position 92 and ending with a TAA codon at position 1379 (Fig. 1). The encoded polypeptide thus contains 429 amino acids and has a predicted molecular mass of ~45 kDa. The predicted amino acid sequence of protein ZAG contains a typical signal peptide and a possible signal cleavage site between amino acids 33 and 34. The C-terminal part of the protein contains all typical features of cell surface proteins from streptococci, such as putative wall-anchoring and membrane-spanning regions as well as the LPXTGX motif (26).

Protein-binding properties of recombinant protein ZAG. Protein ZAG expressed by *E. coli* clone pSZG40 was affinity purified by using immobilized IgG. The protein had a molecular mass of ~45 kDa, as judged by SDS-PAGE (data not shown). This is slightly larger than the calculated molecular mass of the mature protein ZAG but in agreement with the value reported for released Fc receptors from *S. zooepidemicus*

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                                                                                            → S
ATGATTGATTAAAAAGAAGGAAGTAGTATAATAAAAGTGACATTAATAGTTTTTAGTGTCAAAGTTTTTTGAAAAAAGCGAGGGAGGAAAAATGGAAAAAACAAAAACGGTATCCTACTT    120
                                                                                          M E K T K T V S Y F                  10
                                                                                    → α₂M
TTTACGTCAATCAGCTGTTGGTTTAGCTTCAGTGTCAGCAGCCTTTCTGGTTGGAACGTCTTCAGTGGGTGCTCTAGATGCTACAACGGTGTTAGAGCCTACAACAGCCTTCATTAGAGA    240
 L R Q S A V G L A S V S A A F L V G T S S V G A L D A T T V L E P T T A F I R E                                              50
GGCTGTTAGGGAAATCAATCAGCTTAGTGATGACTACGCTGACAATCAAGAGCTTCAGGCTGTTCTTGCTAATGCTGGAGTTGAGGCACTTGCTGCAGATACTGTTGATCAAGCCAAAGC    360
 A V R E I N Q L S D D Y A D N Q E L Q A V L A N A G V E A L A A D T V D Q A K A                                              90
AGCTCTTGACAAAGCAAAGGCAGCTGTTGCTGGTGGTTCAGCTTGATGAAGCAAGACGTGAGGCTTACAGAACAATCAATGCCTTAAGTGATCAGCACGAAAGCGATCAAAAGGTTCAGCT    480
 A L D K A K A A V A G V Q L D E A R R E A Y R T I N A L S D Q H E S D Q K V Q L                                             130
                                                                             → Alb
AGCTCTAGTTGCTGCAGCAGCTAAGGTGGCAGATGCTGCTTCAGTTGATCAAGTGAATGCAGCCATTAATGATGCTCATCAGCTATTGCGGACATTACAGGAGCAGCCTTGTTGGAGGC    600
 A L V A A A A K V A D A A S V D Q V N A A I N D A H T A I A D I T G A A L L E A                                             170
TAAAGAAGCTGCTATCAATGAACTAAAGCAGTATGGCATTAGTGATTACTATGTGACCTTAATCAACAAAGCCAAAACTGTTGAAGGTGTCAATGCGCTTAAGGCAGAGATTTTATCAGC    720
 K E A A I N E L K Q Y G I S D Y Y V T L I N K A K T V E G V N A L K A E I L S A                                             210
    → IgG
TCTACCGAGTTCTGAGGTCATTGACGCAGCAGAACTAACACCAGCCTTGACTAGCTATAAGCTTGTCATCAAGGGAGCAACTTTTTCAGGTGAAACAGCTACTAAGGCAGTAGACGCAGC    840
 L P S S E V I D A A E L T P A L T S Y K L V I K G A T F S G E T A T K A V D A A                                             250
                                                       → IgG
TGTAGCTGAGCAGACCTTCAGAGACTATGCTAATAAAAAATGGTGTAGACGGCGTTTGGGCTTATGATGCTGCCACAAAGACATTTACAGTCACTGAACAGCCTGTAGCTGAGCTATTGA    960
 V A E Q T F R D Y A N K N G V D G V W A Y D A A T K T F T V T E Q P V A E T I E                                             290
GGCGGCAGAATTAACACCAGCTTTGACCACCTATAGGCTTGTTATTAAGGGGGGTTACCTTCTCAGGTGAAACAGCTACCAAGGCAGTAGACGCAGCCACAGCCGAGCAGGCTTTCAGGCA    1080
 A A E L T P A L T T Y R L V I K G V T F S G E T A T K A V D A A T A E Q A F R Q                                             330
                                                   → W
ATACGCGAATGACAATGGCGTTACTGGTGAATGGGCTTATGATGCTGCTACAAAGACATTTACAGTCACTGAGGCTTTAGAGGAGGAGTCCAGCTGAGCCAGAAAAGCCATCAGCTTCTCT    1200
 Y A N D N G V T G E W A Y D A A T K T F T V T E A L E E S P A E P E K P S A S L                                             370
                                                                            → M
TCCGTTGACACCGCTTACACCAGCAACTAAGACAGCACCAGCTAAGCAAAAGGATAAGGAAAAGGCTAAGACTCTTCCAACGACTGGTGAGAAGGCTAATCCATTCTTTTACAGCAGCGGC    1320
 P L P L T P A T K T A P A K Q K D K E K A K T L P T T G E K A N P F F T A A A                                               410
TCTTGCCATTATGGCAGGTGCAGGTGCTTTAGCAGTGACTTCAAAGCGTCAGCAGGACTAAGCAGCGTATTTATGGAAGCATGTTTAGCCTTAGAAGGATGAGATGAGCTTAGTCATAGG    1440
 L A I M A G A G A L A V T S K R Q Q D *                                                                                     429
ACTTTTCTATGATGTAGCCCATTTAAGCATTAGCAGATAAGATAGCAAGAGAAGATCATTAATCAGCCTTTTGCAGTGACTTCAACTGAAGGCTCATACGACGTTTTTGTTAACGTTGTA    1560
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *zag* gene from *S. zooepidemicus* Z5. Underlined nucleotide sequence represents possible promoter signals; double-underlined nucleotide sequence represents the ribosome binding site. Underlined amino acid sequence represents the putative wall-anchoring LPXTGX consensus hexapeptide. S, start of the predicted signal sequence; α₂M, the α₂M-binding domain; Alb, the serum albumin-binding domain; IgG, IgG-binding domains. The putative wall- and membrane-spanning regions are marked with W and M, respectively.

cells (23, 32). It was previously discovered that the IgG-binding activity in protein MIG and protein MAG was combined with the ability to bind α_2M (13, 14) and, in the case of protein MAG, also albumin. The purified protein ZAG was therefore tested in Western blots for the binding of serum albumin and α_2M. Interestingly, protein ZAG bound both of these ligands (data not shown).

Identification of the binding domains. In the N terminus of the mature ~42-kDa protein, there is a unique stretch of 127 amino acids followed by a 50-amino-acid-long region with homology to the albumin-binding domains of other streptococcal receptors. Downstream from this region there are two 70-amino-acid repeats homologous to the IgG-binding domains of type III Fc receptors. On the basis of the sequence analysis and Western blot data, various parts of the *zag* gene were subcloned and expressed as fusion proteins (Fig. 2). Clone pZAG1, which corresponds to the whole protein ZAG, expressed binding of all three ligands. Clone pZAG2, encoding the N-terminal part of the protein (amino acids 30 to 202), expressed only α_2M-binding activity, while clone pZAG3, encoding 170 amino acids (203 to 372) of the C-terminal part of protein ZAG, expressed only IgG-binding activity. To obtain a clone expressing the albumin-binding activity separately, a DNA fragment predicted to encode the albumin-binding activity (amino acids 161 to 212) was PCR amplified and cloned separately. The corresponding clone, pZAG4, indeed expressed albumin-binding activity.

The binding properties of protein ZAG. To characterize the IgG- and albumin-binding properties of protein ZAG, dot blot assays were performed as described in Materials and Methods. Preparations of horse, guinea pig, goat, sheep, cow, rabbit, pig, human, rat, dog, cat, and hen IgGs were applied on NC filters, and the binding of protein ZAG to the different IgG preparations was determined (Fig. 3A). In this assay, all immunoglobulins except those of hen and cat origin were detected by protein

ZAG. A dot blot assay similar to the one used for determination of IgG-binding specificity was also performed with different albumins. Under these conditions, protein ZAG bound serum albumin of human, horse, rat, mouse, and dog origin but not albumin of pig, rabbit, sheep, cow, hen, or goat origin (Fig. 3B).

We also examined the interaction of protein ZAG with different ligands by using a real-time biospecific interaction analysis. The BIAcore data obtained with different IgGs supported the data from the dot blot assays (Table 1). The strongest binding was with horse IgG, with a calculated K_{aff} of 1.0×10^{10} M^{-1}, while the weakest detected interaction was with dog IgG, with a calculated K_{aff} of 1.4×10^{8} M^{-1}. Also with this method, no interaction was detected with hen and cat IgGs. By using the BIAcore system, the binding of HSA and bovine α_2M to immobilized protein ZAG was studied. From analysis of sensorgrams generated from four different concentrations of HSA and α_2M, the binding rate constant (k_a) and the dissociation rate constant (k_d) were determined (Table 1). The calculated K_{aff} values for the interaction with HSA and α_2M were 8.3×10^{7} and 1.4×10^{8} M^{-1}, respectively (Table 1).

Inhibition of α_2M-binding by proteins ZAG, MIG, and MAG. Protein ZAG is the third streptococcal protein that we have described in which a unique N-terminally located domain mediates the binding of α_2M. To investigate the relationship of α_2M binding of these proteins, we examined if they competed for the same or nearby binding site(s) of α_2M in an inhibition assay. The result (Fig. 4) showed that cell lysates of *E. coli* clones expressing the α_2M-binding domains from proteins ZAG, MAG, and MIG fused to MBP inhibited the binding of α_2M to *S. zooepidemicus* cells immobilized in wells of microtiter plates. A control lysate containing the IgG-binding domains of protein ZAG in an analogous construction fused to MBP had only a minor effect on binding.
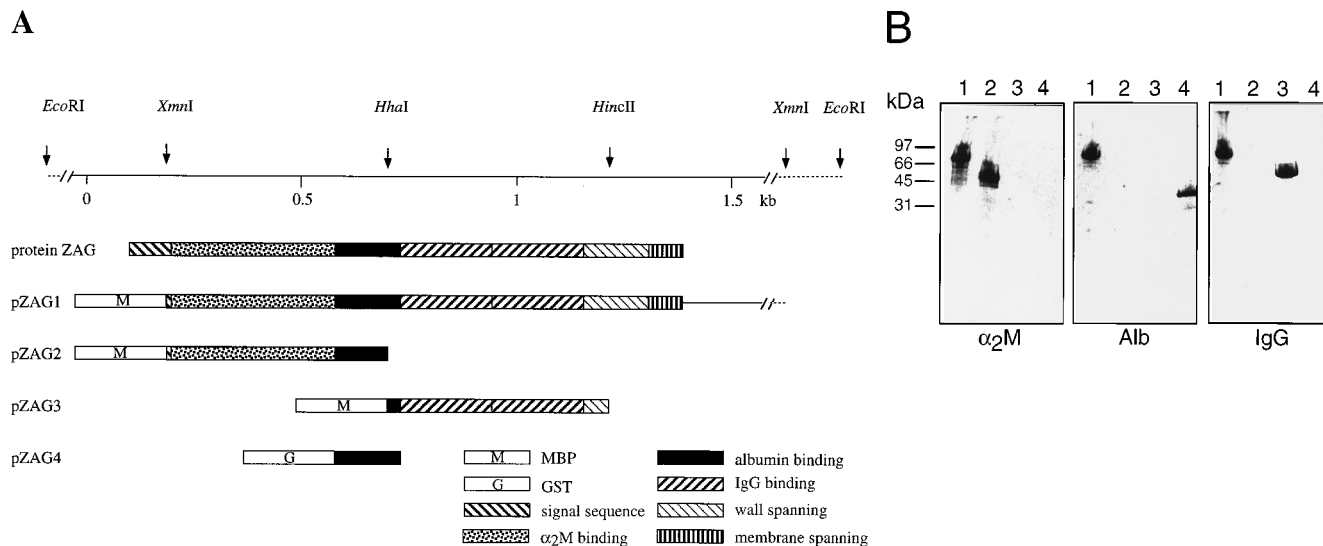
A

B

FIG. 2. Binding domains of protein ZAG. (A) Schematic representation of the protein products encoded by the expression clones pZAG1-4. Restriction sites used in the construction work are indicated. The *Eco*RI sites delineate the 2.9-kb fragment of pSZG40. The MBP and glutathione *S*-transferase (GST) portions are not drawn to scale. (B) Western blot analysis. After separation by SDS-PAGE, the proteins were transferred to NC filters and analyzed for binding of HRP-labelled bovine α_2M, HSA (Alb), and rabbit IgG. Lanes 1 to 4 correspond to lysates from the *E. coli* clones pZAG1 to pZAG4, respectively.

DISCUSSION

The Fc receptors from *S. zooepidemicus* have been reported to be functionally and antigenically distinct from the type III Fc receptors, but so far no detailed information on the structure of Fc receptors from this species has been available (6, 18, 32).

The cloning and sequencing of the *zag* gene revealed that protein ZAG from *S. zooepidemicus* Z5 has many similarities to the type III Fc receptors from group C and G streptococci. An alignment of the binding domains of protein ZAG with the binding domains from similar streptococcal proteins is shown in Fig. 5. The IgG-binding domains from protein ZAG are homologous to the IgG-binding domains in protein G as well as to the corresponding domains in proteins MIG and MAG from *S. dysgalactiae* (Fig. 5A). Alignment of the amino acid sequences, using the single IgG-binding domain in protein MAG as the consensus, reveals an intriguing pattern of amino acid substitutions in the other domains. Compared with the consensus, the IgG-binding domains in ZAG contain patches of amino acid exchanges that specifically relate them to each other and to the four N-terminal domains from protein MIG. Thus, in all of these six domains, amino acid position 22 and positions 26 and 27 have been changed to K and FS, respectively. There are changes also in other positions (e.g., positions 3, 38, 50, and 56) that indicate a specific relationship to both protein MIG and protein G. The evolutionary pathways leading to the variations in the different IgG-binding domains in these four proteins are not immediately apparent. However, as seen in other streptococcal cell surface proteins (11, 30), gene duplication and point mutations together with intergenic recombination could be ways to achieve this variability.

The IgG-binding spectrum of protein ZAG (Fig. 3A and Table 1) confirms a binding pattern similar to the spectra reported earlier for *S. zooepidemicus* (6), although ZAG was reactive also with dog IgG.

Protein ZAG shows an albumin-binding profile (Fig. 3B) similar to those of protein G (20) and the albumin-binding DG12 protein from a bovine group G streptococcus (27). Alignment of the albumin-binding domain of protein ZAG

A

B
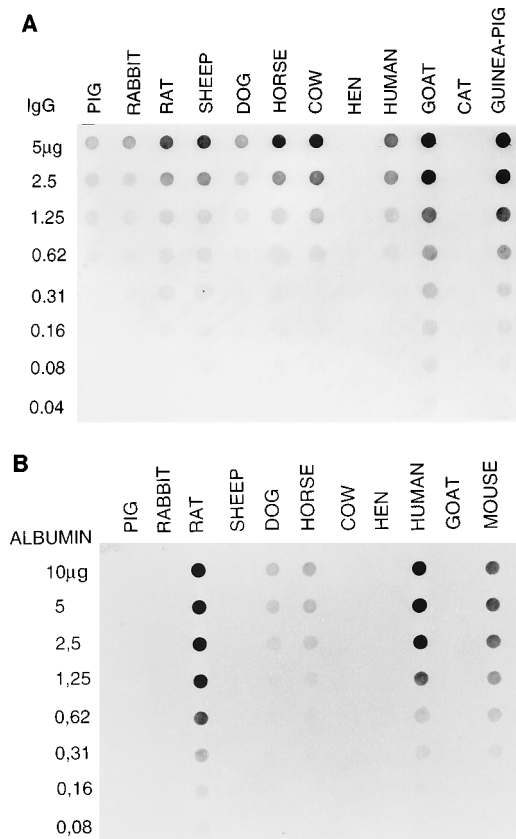
FIG. 3. IgG- and albumin-binding profiles of protein ZAG. Dot blot assays show the reactivity of protein ZAG with IgGs or serum albumins from various species. Protein ZAG in solution was allowed to bind to IgGs or albumins immobilized on an NC filter. (A) Protein ZAG bound to IgGs of various species was detected through its albumin-binding activity by a second round of incubation with HRP-conjugated HSA. (B) Protein ZAG bound to albumins of various species was detected through its Fc-binding activity by a second round of incubation with HRP-labelled goat antibodies.

TABLE 1. Kinetic constants for the binding of different plasma proteins to immobilized protein ZAG[a]

Sample	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_{aff} (M^{-1})	SE	
				k_a	k_d
IgG					
Pig	1.9×10^5	4.7×10^{-4}	4.0×10^8	2.0×10^4	1.6×10^{-6}
Rabbit	4.1×10^5	4.1×10^{-4}	1.0×10^9	3.8×10^4	2.1×10^{-6}
Rat	2.3×10^5	1.3×10^{-3}	1.8×10^8	3.4×10^3	4.1×10^{-6}
Sheep	3.1×10^5	2.0×10^{-4}	1.5×10^9	1.2×10^4	1.0×10^{-6}
Dog	3.3×10^5	2.3×10^{-3}	1.4×10^8	2.0×10^4	1.1×10^{-5}
Horse	2.5×10^5	2.5×10^{-5}	1.0×10^{10}	1.9×10^4	1.2×10^{-6}
Cow	4.8×10^5	2.3×10^{-4}	2.1×10^9	2.6×10^4	9.3×10^{-7}
Human	2.1×10^5	5.6×10^{-4}	3.9×10^8	1.6×10^4	3.3×10^{-6}
Goat	5.1×10^5	1.2×10^{-5}	5.5×10^9	2.4×10^4	1.2×10^{-6}
Guinea pig	3.6×10^5	1.0×10^{-4}	3.6×10^9	2.0×10^4	9.0×10^{-7}
Albumin (human)	4.1×10^4	4.9×10^{-4}	8.3×10^7	1.4×10^3	8.8×10^{-7}
α_2M (cow)	2.5×10^4	1.8×10^{-4}	1.4×10^8	5.1×10^3	8.9×10^{-7}

[a] Using the BIAcore evaluation software, we calculated the kinetic constants for the interaction of different IgGs with protein ZAG from the highest IgG concentrations (40 nM). The kinetic constants for the interaction with HSA and α_2M with protein ZAG were calculated from the average of four different ligand concentrations. k_d was calculated from the first 60 s of the dissociation phase.

with the corresponding domains of other streptococcal proteins also reveals a close evolutionary relationship (Fig. 5B).

Protein ZAG was also found to interact with the plasma proteinase inhibitor α_2M through a unique amino acid sequence (Fig. 5C). Surprisingly, protein ZAG competes for the same, or nearby, binding site(s) as do proteins MIG and MAG in the α_2M molecule, as shown by the inhibition assay (Fig. 4). The N-terminal variability represented by the different α_2M-binding domains implies that these regions are under a higher immunological pressure in comparison with the other binding domains. An alternative explanation of this sequence diversity could of course be that the α_2M-binding domains have evolved through a convergent genetic pathway selected for α_2M binding.

A schematic comparison based on the pairwise amino acid sequence alignment of the various functional domains of proteins MIG, MAG, and ZAG and protein G is shown in Fig. 6. There is a high amino acid sequence homology in the signal sequence, followed by a region of low homology representing the α_2M-binding domains in proteins MIG, MAG, and ZAG and by the functionally undefined region E in protein G (21). Proteins MAG and ZAG also contain what can be considered monomeric forms of the repetitive albumin-binding domains in protein G and the DG12 protein. Proteins G, MIG, MAG, and ZAG contain different numbers of homologous IgG-binding domains, ranging from one domain in protein MAG to five domains in protein MIG. Downstream of the IgG-binding domains, the homology is even more pronounced. The domains suggested to span the cell wall and the membrane show homology in the range of 85 to 100% between the different proteins, although the somewhat shorter cell wall-spanning domain in protein ZAG is only approximately 60% homologous to the corresponding parts in proteins MIG, MAG, and G (Fig. 1 and 6). As judged by the amino acid sequence homologies, there is a clear evolutionary relationship between these proteins. However, analysis of the nucleotide sequence of the 5′ noncoding regions of the genes provides some interesting information. While this part of the genes for proteins G, MIG,
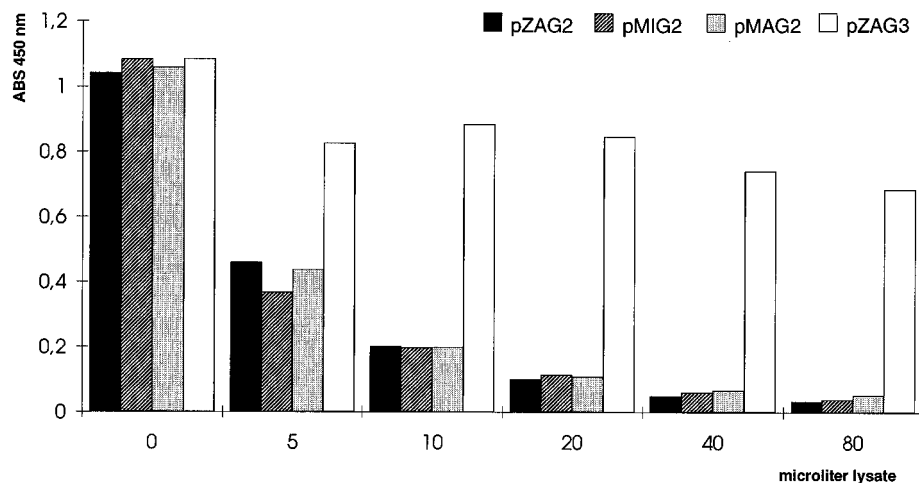

FIG. 4. Inhibition of α_2M binding to *S. zooepidemicus* cells by proteins ZAG, MAG, and MIG. *E. coli* cell lysates containing the α_2M-binding domains from proteins ZAG, MIG, and MAG, expressed as fusion proteins, were tested for their inhibitory effects on α_2M binding to immobilized *S. zooepidemicus* Z5 cells. Clone pZAG2 expressing the α_2M-binding domain of protein ZAG and clone pZAG3 expressing the IgG-binding repeats of protein ZAG, used as a control, are shown in Fig. 2A. For details regarding the construction of clones pMIG2 and pMAG2 expressing the α_2M-binding domains of proteins MIG and MAG, respectively, see references 13 and 14. The mean values of duplicates are shown. The maximum and minimum values were in no case more than 8% different from the mean value.

A

```
                        10        20        30        40        50        60        70
MAG  (243-312)  KPAVIDAPELTPALTTYKLVINGKTLKGETTTKAVDAETAEKAFKQYANENGVDGVWTYDDATKTFTVTE
MIG 5 (489-588)
MIG 4 (419-488)                 IVK  N FS                                    Y E  S
MIG 3 (349-418)                 IVK  N FS          I    A     E        TA      E  S
MIG 2 (279-348)                 IVK  N FS                              TA  N   E  S
MIG 1 (209-278)  A  T            VK  N FS          I  TA     E        TA  N   E  S
ZAG 2 (285-354)  VA ET E  A          R  K V FS  A     A     Q  R      D     T E  A    A
ZAG 1 (212-282)  SS E    A        S     K A FS  A     AV QT RD        K         A    A
SPG 3 (518-587)     E    S   V                                        D
SPG 2 (448-517)     E    S   V                    E   A     V        D         E
SPG 1 (393-447)  --------------- IL                E   A     V        D         E
```

B

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      161
ZAG   DITGAALLEAKEAAINELKQYGISDYYVTLINKAKTVEGVNALKAEILSA
      193
MAG   ALKAAALAKAKADAIEILKKYGIGDYYIKLINNGKTAEGVTALKDEILAS
      248
SPG   TVKSIELAEAKVLANRELDKYGVSDYYKNLINNAKTVEGVKALIDEILAA
      200
DG12  TLKSIKLSEAKEMAIRELDAQGVSDFYKNKINNAKTVEGVVALKDLILNS
      ....  * .** *   *.  *..*.*  . **..**.*** ** . **..
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C

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      34
ZAG   ---ALDATTVLEPTTAFIREAVREINQLSDDYADNQELQAVLANAGVEALAADTVDQAKAALDKA-----KAAVAGV---------------------
      35
MAG   EESTVSPVTVATDAVTTSKEALAIINKLSEDNLNNLDIQEVLAKAGRDILASDSADTIKALLAEV-----TAEVTRLNEEKMARDAVDK---------AI
      31
MIG   LENTITVSAETIPAAVIVPVGL-DTTELQKWYDIANDLVATDNATPGGVFTADSMKALYRLLNDAYDVLESKDYRKYDSQDRIVELVNNLKNTTQSLLPI
      ...  .       ...      ..  ...* .       . .. . . . . ....*. .        *...        . .

ZAG   -------QLDEARREA-YRTINALSDQHES----DQKVQLALVAAAAKVADAA------SVDQVNAAINDAHTAIA------
MAG   AADAAAFSELKDAQLKA-YEDLAKLAADTDL----DLDVAKIINDYTTKVENAK------TAEDVKKIFEESQNEVTRIKTEK
MIG   GVEPVVF---DTTRLNTWYDAANEIVNNSDAYTAESIQPLYKLINDAYDVLESKDYSKYDSQDKVNNLADQLRDAVQAVQLE-
      ....  ..* . . .  .    .       .     .   .*  ..    . ..*.   .. ....
```
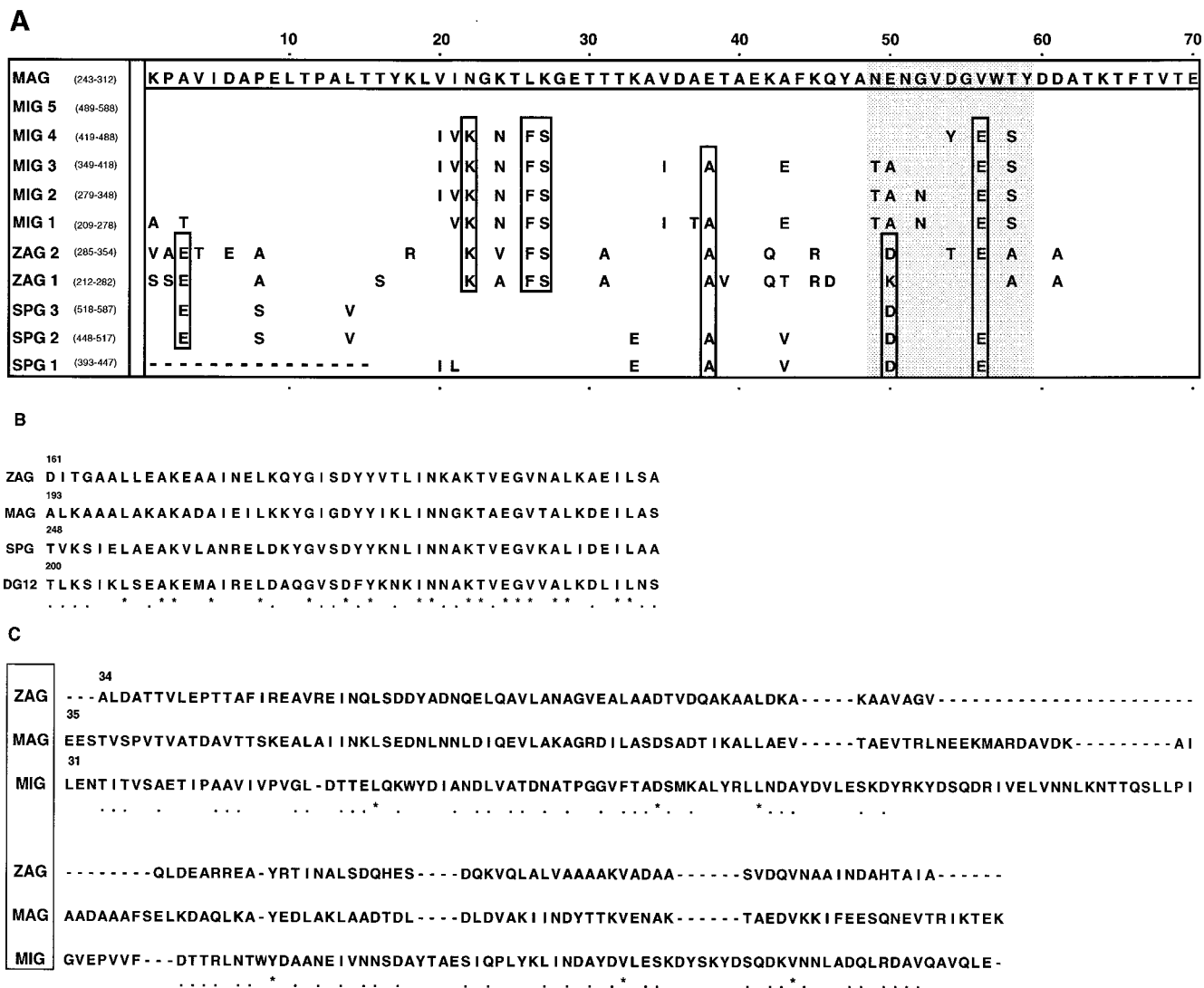
FIG. 5. (A) Alignment of the amino acid sequences of the IgG-binding domains of proteins G (10), MIG (14), MAG (13), and ZAG. The amino acid sequence from the single IgG-binding domain of protein MAG is used as the consensus sequence. The two IgG-binding tandem repeats of protein ZAG, the five tandem repeats of protein MIG, and the three tandem repeats of protein G (SPG) are aligned sequentially, starting with the C-terminal repeats. The numbers in the parentheses indicate the positions of the first and last amino acids of each repeat in the corresponding protein sequence. Changed amino acids are indicated. The boxes indicates positions of amino acid substitutions specifically relating the IgG-binding repeats of protein ZAG to the corresponding repeats in protein MIG and protein G. The position in the respective repeat, corresponding to the 11-amino-acid peptide inhibiting the binding of protein G to IgG as determined by Frick et al. (9), is shadowed. The dashed line indicates the absence of a corresponding region preceding the N-terminal repeat in protein G. (B) Alignment of the amino acid sequences of the albumin-binding domains of proteins G, DG12, MAG, and ZAG. The albumin-binding domain of protein ZAG is defined as the amino acid sequence encoded by the PCR-constructed clone pZAG4 (this work). The albumin-binding activity of protein MAG is defined by Jonsson et al. (13). The parts of the albumin-binding domains of proteins G (1, 4, 19) and DG12 (27) shown in this alignment were those shown to have the best alignment with the albumin-binding domain of protein ZAG. Asterisks indicate perfectly conserved amino acids, and dots indicate well-conserved amino acids. (C) Alignment of the amino acid sequences of the α₂M-binding domains of proteins MIG, MAG and ZAG. Asterisks indicate perfectly conserved amino acids, and dots indicate well-conserved amino acids. The numbering of the amino acids in the sequences and definition of the binding domains of proteins MIG and MAG are from the work of Jonsson and Müller (14) and Jonsson et al. (13), respectively.

and MAG shows pairwise homology in the range of 85 to 99%, the value for the pairwise comparison of the corresponding part of the *zag* gene with the other three genes is in the range of 36 to 45%. Partial sequence data show a similar result for the 3′ noncoding regions of the genes (data not shown). The parental strains for proteins G, MIG, and MAG all belong to the species *S. dysgalactiae*, while *S. zooepidemicus* is a subspecies of *S. equi* which is taxonomically distinct from *S. dysgalactiae* (3, 25). Taken together, these data indicate that the *zag* gene was obtained after the two species separated.

On the basis of the sequence homologies, and taking into account the α₂M binding in proteins MIG, MAG, and ZAG, we suggest that they be regarded as members of a family of protein G-related proteins encoded by *grp* genes. A model for the evolution of this family of genes must await sequence data from a greater number of genes. Recently, de Château and Björck (8) reported on an albumin-binding protein from *Peptostreptococcus magnus* called protein PAB, with a mosaic organization which probably arose through intergenic interspecies recombination of functional domains. Interestingly, this protein contains a short albumin-binding domain with homology to the streptococcal albumin-binding domains of the pro-
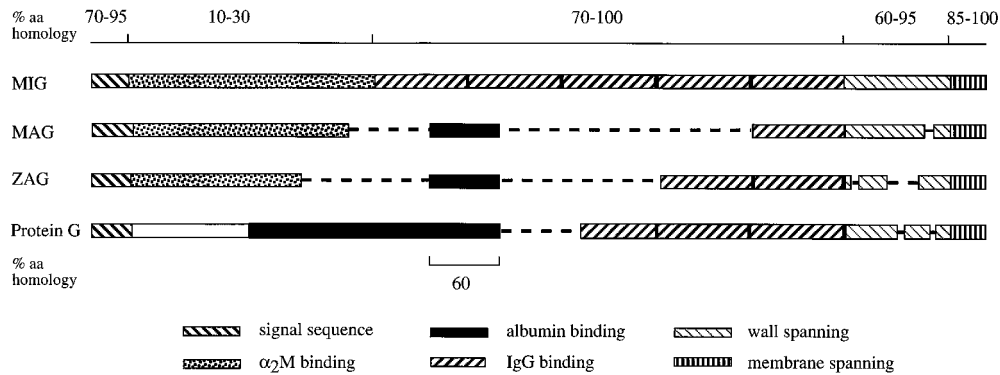
FIG. 6. Comparison of proteins G, MIG, MAG, and ZAG, with their different functional domains indicated. Protein MIG (14), protein MAG (13), protein G (10, 21), and protein ZAG are schematically presented, and the approximative percentage amino acid homologies derived from pairwise alignment of the different domains are indicated. The percent homology of the albumin-binding domains is based on the part of the albumin-binding domain from protein G shown in Fig. 5B. The white box in protein G corresponds to the alanine-rich E region, the biological function of which is not known (21). Gaps, indicated by dashed lines, have been introduced in the alignment in order to position regions with high homology on top of each other.

teins discussed in this work. The importance of proteins MIG, MAG, and ZAG in infection and host defense is not known, but Valentin-Weigand et al. (29) have published interesting data showing that the binding of proteinase-complexed α_2M to animal group C streptococci inhibits phagocytosis in vitro. This finding indicates that proteins of this type have functional similarities to the streptococcal M proteins.

ACKNOWLEDGMENTS

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