Binding of α_2 -macroglobulin to GRAB (Protein G-related α_2 -macroglobulin-binding protein), an important virulence factor of group A streptococci, is mediated by two charged motifs in the ΔA region

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GRAB (Protein G-related α_2 M-binding protein) is a surface protein of group A streptococci and exhibits high affinity for α_2 -macroglobulin (α_2 M), a broad-range protease inhibitor. It is the sole α_2 M-binding protein of group A streptococci that has been shown to promote bacterial virulence in a mouse model of skin infection. The binding site for α_2 M was predicted to be in the N-terminal A domain of GRAB. In the present study, the α_2 M-binding domain was first narrowed down to 34 amino acids (amino acids 34–67) using variable truncated N-terminal GRAB fusion proteins. The sequence of the identified domain was used to design overlapping synthetic peptides of different sizes, which were then immobilized on a membrane and assayed for their α_2 M-binding activity. The peptide screening revealed two binding motifs of ten amino acids length, located in the ΔA (N-terminal part of the A domain) region (amino acids 34–67) with the sequences PRIIPN-GGTL (amino acids 41–50) and NAPEKLALRN (amino acids 56–65) respectively. These motifs were used for systematic muta-

tional analysis by generating synthetic peptides containing individual amino acid substitutions at every position of the mapped binding regions. The results indicated a critical role for the arginine residue at position 42 in the first binding domain and at position 64 in the second binding region. Validation of arginine residues as the critical amino acids for α_2 M binding was achieved by site-directed mutagenesis and binding assays. Competitive inhibition assays with GRAB containing amino acid substitutions R42G (Arg⁴² → Gly), R64G and R42G/R64G indicated differential contribution of the arginine residues at positions 42 and 64 to α_2 M-binding activity and, thus, their involvement in GRAB-induced virulence.

Key words: α_2 -macroglobulin (α_2 M), motif, peptide, Protein G-related α_2 -macroglobulin-binding protein (GRAB), streptococcus, virulence.

INTRODUCTION

Group A streptococci (GAS; *Streptococcus pyogenes*) are important human pathogens that are commonly associated with mild and self-limiting diseases such as pharyngitis, pyoderma or asymptomatic colonization. Nevertheless, GAS have the potential to cause life-threatening invasive diseases like necrotizing fasciitis, bacteraemia, toxic shock syndrome and rheumatic heart disease [1,2]. In view of the resurgence of severe streptococcal invasive infections since the early 1980s [3–7], elucidation of the mechanisms that enable bacteria to invade the host and survive its defence mechanisms, including phagocytosis or complement-mediated cell lysis, is pivotal to a better understanding of GAS diseases [1,8–10].

The surface protein GRAB [Protein G-related α_2 M (α_2 -macroglobulin)-binding protein] is one of the GAS components promoting virulence [11] and was originally identified by the high degree of homology of its N-terminal part with the α_2 M-binding E domain of Protein G from group C and G streptococci, indicating a similar α_2 M-binding mechanism in both proteins [12–15]. GRAB is a protein with a molecular mass of 22.8 kDa, consisting of a cell-wall-anchoring region with a typical Gram-positive membrane anchor LPXTG motif [8,16], a varying number of 28-amino-acid-long repeats and a highly conserved, functional N-terminal domain responsible for the interaction with the broad-range protease inhibitor α_2 M [12]. α_2 M is a 725 kDa homotetrameric

plasma glycoprotein capable of inhibiting proteases of all classes and specificities through a unique mechanism involving steric entrapment and covalent binding [17–20]. The *grab* gene is present in almost all GAS isolates and contributes to bacterial virulence, as shown recently in a murine skin model of GAS infection [11,12]. Recruitment of a broad-range protease inhibitor such as α_2 M by the bacteria has been proposed as a mechanism leading to the protection of bacterial surface structures, such as the antiphagocytic M-protein, from proteolytic degradation [12,21,22]. Furthermore, depletion of the surrounding protease inhibitors probably leads to an increase in the amount of free proteases and enhances tissue destruction during the infection process [9,23,24]. Thus interaction with protease inhibitors, such as α_2 M, may enable bacteria to protect their surface structures and facilitate progressive dissemination in the tissue [11]. The fact that neither GAS nor human pathogenic group G and C streptococci bind the electrophoretically fast form of α_2 M (f- α_2 M) that had already been complexed with proteases supports this hypothesis [25]. Both pathogens only bind the electrophoretically slow form of α_2 M (s- α_2 M) with protease inhibitory activity [14,26–28]. Neither GRAB protein itself nor Protein G exhibits proteolytic activity, altering the conformational status of the bound plasma protein from s- α_2 M to f- α_2 M as demonstrated in previous studies [12]. Thus surface-recruited α_2 M remains active and provides the bacterium with a mechanism to interact with foreign or its own proteases [12,14,15].

Abbreviations used: ΔA , N-terminal part of the A domain; α_2 M, α_2 -macroglobulin; f- α_2 M, electrophoretically fast form of α_2 M; s- α_2 M, electrophoretically slow form of α_2 M; BAEE, *N*_α-benzoyl-L-arginine ethyl ester hydrochloride; GAS, group A streptococci; GST, glutathione S-transferase; (r)GRAB, (recombinant) Protein G-related α_2 M-binding protein.

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In the present study, the GRAB- α_2 M interaction was analysed to map the minimal binding motif(s) and critical amino acid(s) of GRAB mediating the high-affinity interaction with α_2 M. Analysis of spot-synthesized synthetic peptides of GRAB and competitive inhibition experiments with recombinant GRAB derivatives identified two binding motifs, **PR**⁴²IIPNGGTL and **NAPEKLALR**⁶⁴N, in the Δ A (N-terminal part of the A domain) region (amino acids 34–67) of GRAB. Individual amino acid substitutions at every position in the motifs and competitive inhibition experiments using the mutated recombinant GRAB derivatives rGRAB⁴², rGRAB⁶⁴ and rGRAB^{42/64} demonstrated that arginine residues are critical for the protein–protein interaction and, hence, have a pivotal role in the GRAB-induced virulence of GAS.

EXPERIMENTAL

Bacterial strains, growth conditions and protein purification

S. pyogenes strains were grown in Todd–Hewitt broth (Invitrogen, Karlsruhe, Germany) supplemented with 1% yeast extract (Difco, Heidelberg, Germany) (referred to as THY) under static conditions at 37 °C or on blood agar plates (Becton Dickinson, Heidelberg, Germany). Epicurian Coli™ XL1-Blue cells as the host for recombinant pGEX-6P-1 (Amersham Biosciences) were grown in Luria–Bertani medium or on Luria–Bertani agar with ampicillin (100 µg/ml). The medium of *Escherichia coli* M15-[pREP4] containing recombinant pQE30 (Qiagen) was supplemented with 100 µg/ml ampicillin and 25 µg/ml kanamycin. Expressions of GST (glutathione S-transferase)- and His-tagged fusion proteins were induced with 1.5 mM isopropyl β -D-thiogalactoside (Sigma–Aldrich) after the culture reached an attenuation (D_{600}) of 0.6 and growth continued for 5 h at 30 °C. Recombinant His-tagged GRAB (rGRAB) and its derivatives rGRAB⁴², rGRAB⁶⁴ and rGRAB^{42/64} were purified by affinity chromatography under native conditions on Ni²⁺-nitrilotriacetic acid agarose according to the general guidelines of QIAexpressionist™ (Qiagen). GST fusion proteins rGST- Δ A, rGST-A, rGST-AR1 and rGST-AR2 were purified by affinity chromatography using glutathione–Sephacrose 4B (Amersham Biosciences) according to the manufacturer's instructions.

Human plasma protease inhibitor α_2 M

The human plasma protease inhibitor α_2 M (M6159), purchased from Sigma–Aldrich, was added to 0.02 M Tris/0.13 M glycine (pH 8.0) containing 0.08 M trehalose and freeze-dried. According to the manufacturer, 1 mg of α_2 M inhibits at least 10 µg of trypsin with an activity of 10 000 BAEE units/mg of protein (where BAEE stands for N_α -benzoyl-L-arginine ethyl ester hydrochloride). The trypsin-binding activity of α_2 M was validated by an assay on the basis of the ability of the protein to protect the entrapped trypsin (Sigma–Aldrich) from inactivation by soya-bean trypsin inhibitor (Sigma–Aldrich), using BAEE (Sigma–Aldrich) as the substrate [28]. Briefly, purified α_2 M was incubated for 7 min at 30 °C with increasing molar ratios of trypsin (Sigma–Aldrich). The proteolytic activity of the remaining trypsin that was not entrapped by α_2 M was inhibited by adding double the molar amount of soya-bean trypsin inhibitor (Sigma–Aldrich). Activity of the remaining trypsin was determined by adding BAEE as substrate for trypsin at a final concentration of 2.5 mg/ml to the reaction and monitoring the ΔA_{253} . Trypsin alone and trypsin/soya-bean trypsin inhibitor were used as controls. In addition, the percentages of slow- and fast-forms of α_2 M in the sample were determined by SDS/PAGE and subsequent densitometric analysis of the different bands. α_2 M preincubated with

Figure 1 SDS/PAGE analysis of α_2 M before and after trypsin treatment

Lane 2 (α_2 M) mainly consists of the slow-form α_2 M with small amounts of the fast form (double bands at 100 kDa mass). In contrast, the trypsin-pretreated control (α_2 M-T) in lane 3 shows the electrophoretically fast-form α_2 M. Molecular markers are shown in lane 1.

trypsin was used as a control (Figure 1). A comparison of the different bands revealed that the protein purchased from Sigma–Aldrich was mainly a slow form (s- α_2 M), non-protease-complexed α_2 M (82%). The amount of f- α_2 M (fast-form α_2 M) detected was less than 20%.

Recombinant DNA techniques and PCR

Chromosomal DNA from the *S. pyogenes* strain A82 was prepared with Genomic-tip 100/G columns (Qiagen) according to the manufacturer's instructions and used as a template for PCR amplification of the *grab* gene. The *grab* sequence of GAS isolate A82 is identical with the *grab* sequence of *S. pyogenes* reference strain SF 370. PCR was performed with approx. 50 ng of chromosomal DNA as a template using *Taq* DNA polymerase (Qiagen) or *Pfu* DNA polymerase (Stratagene) under the buffer conditions recommended by the manufacturer. The PCR products were purified by gel extraction using the QIAquick PCR Purification kit (Qiagen). Plasmid DNA was isolated using QIAprep Spin Plasmid kit (Qiagen). *T4* DNA ligase and the restriction enzymes were purchased from New England Biolabs. The transformation of recombinant plasmid DNA in *E. coli* was achieved by electroporation [30].

Cloning procedures

grab DNA fragments were amplified with oligonucleotides incorporating a *Bam*HI restriction site at the 5'-end and a *Kpn*I or *Eco*RI restriction site at the 3'-end. Oligonucleotides were designed from the *grab* sequence of the *S. pyogenes* strain SF 370 (A.T.C.C. 700294; GenBank® accession no. GI:4589078). PCR products were digested with *Bam*HI–*Kpn*I or *Bam*HI–*Eco*RI respectively and ligated into similarly prepared expression vectors to allow the in-frame expression of GST- or His-tagged fusion proteins. The oligonucleotides, their features and the resulting *grab* DNA fragments generated by PCR are listed in Table 1.

Site-directed mutagenesis of *grab*

Site-directed mutagenesis of *grab* was performed by the method of Higuchi et al. [31] by incorporating single-base mismatches between the synthetic oligonucleotide and the template by PCR amplification. Each mutant *grab* DNA was generated in a two-step procedure. First, the overlapping DNA fragments designated as PCR1/PCR2 and PCR3/PCR4, containing the desired base-pair substitution at the selected position, were amplified by PCR (Table 1). Secondly, a fusion PCR was performed to generate the entire mutated DNA fragment from the overlapping fragments

Table 1 Sequence, name and features of the synthetic oligonucleotides used for the generation of recombinant His-tagged GRAB proteins and GST-tagged rGST- Δ A, rGST-A, rGST-AR1 and rGST-AR2

Site-directed mutagenesis resulting in the substitution of Arg⁴² was performed using oligonucleotides containing single-point mutations (primers 8–11). The codon CGA encoding an arginine residue was changed to GGA encoding a glycine. Insert DNA for pQGR-42 (rGRAB⁴²) was PCR-amplified by a fusion PCR using PCR products 1 and 2 with external primers 1 and 2. The DNA for pQGR-64 (rGRAB⁶⁴) was generated using PCR products 3 and 4 with external primers 1 and 2. The double-mutant pQGR-42/64 (rGRAB^{42/64}) was generated by the method used for the generation of pQGR-64 using pQGR-42 as template DNA.

No.	Name	Oligonucleotide sequence	Nucleotide position in <i>grab</i>	Primer	Clone	Protein
1	<i>grab</i> -BamHI	5'-CGCGGATCCGTTGACTCACCTATCGAACAGCC-3'	100–122			
2	<i>grab</i> -KpnI	5'-CGGGGTACCTAGGTATTAACGTTTTGTCTTTCTTT-3'	546–520	1/2	pQGR	rGRAB
3	Δ A-EcoRI	5'-CGGAATTCCTTCTTACGTAATGCCAGTTTTTC-3'	201–175	1/3	pG Δ A	rGST- Δ A
4	A-EcoRI	5'-CGGAATTCACCTTGCTGCTTCTATAGCTGTGCG-3'	273–251	1/4	pGA	rGST-A
5	R1-EcoRI	5'-CGGAATTCAGCAGCGTTATCCGCTTTAAAC-3'	357–334	1/5	pGAR1	rGST-AR1
6	R2-EcoRI	5'-CGGAATTCAGCAGCGTTATCTGATTGAAC-3'	441–418	1/6	pGAR2	rGST-AR2
7	<i>grab</i> -F	5'-ATGGGAAAGAAATAAAAGTGAATGC-3'	1–27			
8	MI-F	5'-CCTATCGAACAGCCT($\frac{5}{6}$)GAATTATCCAAATGGCGGAAC-3'	109–146	7/9	PCR 1	
9	MI-R	5'-CCATTGGGAATAATC($\frac{5}{6}$)AGGCTGTTCCGATAGGTGAG-3'	140–105	8/2	PCR 2	
10	MII-F	5'-CAGAAAACTGGCATT($\frac{5}{6}$)GTAATGAAGAAAGAGCCATTGATG-3'	173–214	1/11	PCR 3	
11	MII-R	5'-GCTCTTTCTTATTAC($\frac{5}{6}$)TAATGCCAGTTTTCTGGAG-3'	206–170	10/2	PCR 4	

PCR1/PCR2 and PCR3/PCR4 using the oligonucleotides *grab*-BamHI and *grab*-KpnI (Table 1). The mutated DNA fragments were cloned in pGEM-T Easy under conditions recommended by the distributor (Promega) and, finally, after digestion with BamHI–KpnI, they were subcloned into the similarly prepared expression plasmid pQE30. The fusion PCR consisted of 2 cycles including 60 s of denaturation at 94 °C, 60 s of annealing at 70 °C and 120 s of extension at 72 °C, followed by 30 cycles including 60 s of denaturation at 94 °C, 60 s of annealing at 55 °C and 90 s of extension at 72 °C. The chromosomal DNA of the *S. pyogenes* strain A82 was used as a template. The oligonucleotides, the introduced point mutations and the resulting *grab* sequences are listed in Table 1. To generate the insert DNA of the double-mutant pQGR42/64 with single-point mutations at nucleotide positions 124 and 190, the plasmid pQGR42 was used as a template DNA by the method used for the generation of pQGR64. This strategy resulted finally in the expression of rGRAB⁴², rGRAB⁶⁴ and rGRAB^{42/64}. The integrity of the mutated *grab* sequences was confirmed by DNA sequence analysis using ABI PRISM dye terminator sequencing (PerkinElmer).

Anti-GRAB antiserum

Antiserum against rGRAB was generated by Eurogentech (Seraing, Belgium). Rabbits were immunized intradermally with 50 μ g of purified rGRAB encoded by pQGR in 1 ml of a 1:1 emulsion of antigen and complete Freund's adjuvant. The animals were boosted with 50 μ g of rGRAB and incomplete adjuvant on days 14, 28 and 56 by the standard method. Polyclonal anti-rGRAB IgG of the derived serum fractions was purified using Protein A–Sepharose (Amersham Biosciences) as recommended by the distributor.

Electrophoresis and blot-overlay assay

Purified fusion proteins were subjected to SDS/PAGE (12% gel) by the method of Laemmli [32]. The gels were then either stained with Coomassie Brilliant Blue or transferred on to a nylon membrane (Immobilon-P; Millipore) using a semi-dry blotting system (Bio-Rad) [33]. Binding of purified human α_2 M (Sigma–Aldrich) to rGRAB and its derivatives was examined by the blot-overlay assay. Membranes were first blocked with 10 mM PBS containing 5% (w/v) skimmed milk for 1 h at room temperature

(21 °C) and incubated for an additional hour with α_2 M (4 μ g of α_2 M/ml of PBS). The binding of α_2 M to GRAB protein was detected with rabbit polyclonal IgG anti-human α_2 M (Sigma–Aldrich; 1:1000-diluted in PBS). Rabbit polyclonal IgG anti-rGRAB (1:500-diluted in PBS) was used as a control. Detection was performed by goat peroxidase-conjugated anti-rabbit IgG antibody (Sigma–Aldrich; 1:5000-diluted in PBS) followed by incubation with the substrate solution of 4-chloro-1-naphthol (1 mg/ml) and 0.1% H₂O₂ in 10 mM PBS.

Radiolabelling of α_2 M with ¹²⁵I and competitive inhibition assay

Radiolabelling of the human protease inhibitor α_2 M (Sigma–Aldrich) was performed using carrier-free ¹²⁵I (Amersham Biosciences) by the chloramine T method with a specific activity of approx. 1 mCi/mg of protein [34,35]. Binding of ¹²⁵I-labelled α_2 M to *S. pyogenes* was assessed as described previously [11,15,27]. In competitive inhibition experiments, the binding of 12 ng of ¹²⁵I-labelled α_2 M to 1.5×10^8 c.f.u./300 μ l of the *S. pyogenes* strains A6 and KTL3 grown at 37 °C in THY to stationary phase was determined in the presence of increasing concentrations of unlabelled purified rGRAB and its derivatives. Recombinant GRAB, its derivatives and mutated GRAB were used at concentrations ranging from 6.6 ng/50 μ l of PBS up to 100 μ g/50 μ l of PBS. The iodination of α_2 M neither influenced the conformational status of the protein as demonstrated by Müller and Rantamäki [15] nor the GRAB-binding ability as shown by the blot-overlay analysis.

Preparation of spot-synthesized peptides on membranes and analysis of α_2 M binding

The A region of GRAB (amino acids 34–91) was predicted to be the α_2 M binding domain. To map the binding site precisely, the amino acid sequence of the A domain was divided into 44 overlapping peptides, consisting of 15 amino acids each, with an offset of one amino acid per peptide spot [36]. The initial sequence (spot 1) was V³⁴DSPIEQPRIIPNGG⁴⁸. The peptides were synthesized as an array of spots on an aminopropylated cellulose membrane (AIMS Scientific Products, Braunschweig, Germany) as described previously [37,38] and analysed for their α_2 M-binding activity in a protein overlay assay using native human α_2 M (Sigma–Aldrich) as a ligand (40 μ g/ml α_2 M). The two α_2 M

Table 2 Sequences of the synthetic membrane-spotted peptides depicted in Figures 5(B) and 5(E)

Spot no.	Synthetic peptides (Figure 5B)	Spot no.	Synthetic peptides (Figure 5E)
1	IIPNGGTL	1	TNLLGNAPE
2	IPNGGTLT	2	NLLGNAPEK
3	PNGGTLTN	3	LLGNAPEKL
4	NGGTLTNL	4	LGNAPKLA
5	GGTLTNLL	5	GNAPEKLAL
6	RIIPNGGTL	6	NAPEKLALR
7	IIPNGGTLT	7	LTNLLGNAPE
8	IPNGGTLTN	8	TNLLGNAPEK
9	PNGGTLTNL	9	NLLGNAPEKL
10	NGGTLTNLL	10	LLGNAPEKLA
11	GGTLTNLLG	11	LGNAPKLA
12	PRIIPNGGTL	12	GNAPEKLALR
13	RIIPNGGTLT	13	NAPEKLALRN
14	IIPNGGTLTN	14	TLTNLLGNAPE
15	IPNGGTLTNL	15	LTNLLGNAPEK
16	PNGGTLTNLL	16	TNLLGNAPEKL
17	NGGTLTNLLG	17	NLLGNAPEKLA
18	GGTLTNLLGN	18	LLGNAPEKLAL
19	QPRIIPNGGTL	19	LGNAPKLA
20	PRIIPNGGTLT	20	GNAPEKLALRN
21	RIIPNGGTLTN	21	NAPEKLALRNE
22	IIPNGGTLTNL	22	GTTLNLLGNAPE
23	IPNGGTLTNLL	23	TLTNLLGNAPEK
24	PNGGTLTNLLG	24	LTNLLGNAPEKL
25	NGGTLTNLLGN	25	TNLLGNAPEKLA
26	GGTLTNLLGNA	26	NLLGNAPEKLAL
27	EQPRIIPNGGTL	27	LLGNAPEKLALR
28	QPRIIPNGGTLT	28	LGNAPKLA
29	PRIIPNGGTLTN	29	GNAPEKLALRNE
30	RIIPNGGTLTNL	30	NAPEKLALRNEE
31	IIPNGGTLTNLL	31	GGTLTNLLGNAPE
32	IPNGGTLTNLLG	32	GTTLNLLGNAPEK
33	PNGGTLTNLLGN	33	TLTNLLGNAPEKL
34	NGGTLTNLLGNA	34	LTNLLGNAPEKLA
35	GGTLTNLLGNAP	35	TNLLGNAPEKLAL
36	IEQPRIIPNGGTL	36	NLLGNAPEKLALR
37	EQPRIIPNGGTLT	37	LGNAPKLA
38	QPRIIPNGGTLTN	38	LGNAPKLA
39	PRIIPNGGTLTNL	39	GNAPEKLALRNEE
40	RIIPNGGTLTNLL	40	NAPEKLALRNEER
41	IIPNGGTLTNLLG	41	NGGTLTNLLGNAPE
42	IPNGGTLTNLLGN	42	GGTLTNLLGNAPEK
43	PNGGTLTNLLGNA	43	GTTLNLLGNAPEKL
44	NGGTLTNLLGNAP	44	TLTNLLGNAPEKLA
45	GGTLTNLLGNAPE	45	LTNLLGNAPEKLAL
46	PIEQPRIIPNGGTL	46	TNLLGNAPEKLALR
47	IEQPRIIPNGGTLT	47	NLLGNAPEKLALRN
48	EQPRIIPNGGTLTN	48	LGNAPKLA
49	QPRIIPNGGTLTNL	49	LGNAPKLA
50	PRIIPNGGTLTNLL	50	GNAPEKLALRNEE
51	RIIPNGGTLTNLLG	51	GNAPEKLALRNEER
52	IIPNGGTLTNLLGN	52	NAPEKLALRNEERA
53	IPNGGTLTNLLGNA	53	PNGGTLTNLLGNAPE
54	PNGGTLTNLLGNAP	54	NGGTLTNLLGNAPEK
55	NGGTLTNLLGNAPE	55	GGTLTNLLGNAPEKL
56	GGTLTNLLGNAPEK	56	GTTLNLLGNAPEKLA
57	PIEQPRIIPNGGTL	57	TLTNLLGNAPEKLAL
58	IEQPRIIPNGGTLT	58	TNLLGNAPEKLALRN
59	EQPRIIPNGGTLTN	59	NLLGNAPEKLALRNEE
60	QPRIIPNGGTLTNL	60	LLGNAPEKLALRNEE
61	PRIIPNGGTLTNLL	61	LGNAPKLA
62	RIIPNGGTLTNLLG	62	GNAPEKLALRNEER
63	IIPNGGTLTNLLGN	63	GNAPEKLALRNEERA
64	IPNGGTLTNLLGNA		NAPEKLALRNEERAI
65	PNGGTLTNLLGNAP		
66	NGGTLTNLLGNAPE		
67	GGTLTNLLGNAPEK		
68	GTTLNLLGNAPEKL

Figure 2 A model and sequence of GRAB

(A) Schematic representation of GRAB protein (accession no. GI: 4589078). Functional domains are (Ss) signal sequence, (A) α_2 M-binding domain with the Δ A region shown hatched, (R) repeat regions, (W) cell-wall attachment site and (M) membrane anchor. (B) Amino acid sequence of GRAB protein. The A region is in bold type and the Δ A region is underlined.

binding sites detected by that assay spanned parts of the 15-amino-acid regions P⁴¹RIIPNGGTLTNLLG⁵⁵ and T⁵¹NLLGNAPEKLALRN⁶⁵. These parts were subsequently divided again into synthetic, overlapping peptides of variable length and were chemically synthesized as above. To define the minimal binding motif, the region corresponding to amino acids 36–61 was divided into 68 overlapping synthetic peptides with an offset of one amino acid each. The membrane consisted of eight series of peptides with lengths varying from 8 up to 15 amino acids per spot (Table 2). The second region corresponded to amino acids 45–70 and was divided into 63 overlapping peptides, consisting of seven series of peptides with lengths varying from 9 up to 15 amino acids per spot (Table 2). In addition, peptide spot membranes were generated for systematic mutational analysis. The membranes contained decapeptides of the two proposed binding domains with individual amino acid substitutions at each position of the original peptide sequences PRIIPNGGTL and NAPEKLALRN. Detection of binding was performed by the blot-overlay assay as described previously [38]. Peptide-specific signals were evaluated by densitometric analysis and the relative intensity of the spots was determined using the EASY Win32 analysis software (Herolab).

RESULTS

α_2 M binds to the Δ A domain of GRAB

The N-terminal part of the A domain, designated Δ A (Figure 2), was predicted to be the preferential binding site for α_2 M [12]. Based on the previous results, five recombinant GRAB derivatives were constructed and produced as GST fusion proteins to narrow down the binding site for α_2 M in GRAB (Figure 3A). The Δ A fragment, comprising amino acids 34–67, represents the N-terminal part of GRAB and exhibits 71 % identity to the E domain of Protein G [12]. All recombinant proteins were separated by SDS/PAGE (Figure 3B) and functionally analysed for their α_2 M-binding activity under reducing conditions in blot-overlay assays (Figure 3C). Immunoblots with anti-GRAB IgG antibodies (Figure 3D) and anti- α_2 M IgG antibodies (Figure 3E) were used as controls. The binding of α_2 M to purified GST protein was tested negatively by Western-blot analysis and also by using ¹²⁵I-labelled α_2 M as a ligand (results not shown). The results revealed that each of the rGRAB derivatives was capable of binding α_2 M. These results suggest that the α_2 M-binding motif is located in the Δ A domain of GRAB, whereas the C-terminal part of the A domain

Figure 3 Immunoreactivity and binding of α_2 M to recombinant GRAB derivatives

(A) Recombinant GRAB fusion proteins. (B) Coomassie Blue stain of the recombinant GRAB derivatives. (C–E) Immunoblot analysis of GRAB derivatives using (C) anti-GRAB IgG, (D) α_2 M and (E) anti- α_2 M IgG as the ligands respectively. Lane 1, rGRAB; lane 2, rGST- Δ A; lane 3, rGST-A; lane 4, rGST-AR1; lane 5, GST-AR2; lane 6, GST; and lane 7, α_2 M. (F) Competitive inhibition of the binding of 125 I- α_2 M to *S. pyogenes* isolates KTL3 and A6 by rGST- Δ A (◆) or rGST-A (■).

and the repeat regions did not play any role in the α_2 M-binding activity of GRAB. Recombinant GST that was used as a negative control interacted neither with anti-GRAB antibodies (Figure 3D) nor with α_2 M (Figure 3C). To compare the binding of native and trypsin-modified forms of α_2 M to the bacterial surface protein GRAB, additional binding assays were performed using α_2 M (Sigma–Aldrich) and trypsin-pretreated f- α_2 M (molar ratio α_2 M/trypsin: 1:2, 1:4 and 1:8) as ligands. The high molar trypsin ratio was used to avoid any residual s- α_2 M. Western-blot analysis did not reveal any binding of trypsin-pretreated f- α_2 M to rGRAB, whereas untreated α_2 M exhibited a strong binding ability (results not shown). α_2 M separated by SDS/PAGE and analysed by

Western blotting using rGRAB as a ligand revealed no binding activity. The results indicate that the interaction between both proteins strongly depends on the conformational status of α_2 M and only native s- α_2 M is capable of interacting with the bacterial surface protein GRAB.

The function of the Δ A region of GRAB as an α_2 M-binding site was confirmed in competitive inhibition assays. The binding of radiolabelled 125 I- α_2 M was assayed using two different *S. pyogenes* isolates KTL3 (M1 serotype) and A6 (M3 serotype) in the presence of increasing concentrations of rGST- Δ A and rGST-A. Both strains tested positive for α_2 M binding in previous experiments (results not shown), and GRAB was shown to be

Figure 4 Determination of putative α_2 M-binding motifs in the A domain of GRAB

(A) The complete amino acid sequence of domain A. (B) Spot-membrane analysis for α_2 M-binding activity of the 58 amino acids, divided into 44 overlapping peptides of 15 amino acids each, with an offset of one amino acid each. (C) Serial numbers and sequences of the spots detected positive for binding to α_2 M.

the only α_2 M-binding protein expressed by KTL3 [12]. The results indicated that both rGST- Δ A and rGST-A were capable of inhibiting the binding of 125 I- α_2 M to GAS by approx. 80%, thus confirming the role of Δ A as the binding region for α_2 M (Figure 3F). The inhibition of not more than 80% is probably due to fusion with GST. With His-tagged rGRAB, more than 97% inhibition could be achieved (results not shown).

α_2 M binding to spot-synthesized peptides deduced from the A region of GRAB

For a fine mapping of the binding site for α_2 M, the A region of GRAB was divided into 44 overlapping peptides, each consisting of 15 amino acids, with an offset of one amino acid each. These immobilized peptides were analysed for their ability to bind α_2 M. The blot-overlay assay revealed two reacting peptide series located between spots 7–10 and spots 17–18 (Figure 4). These two peptides represent amino acid sequences of Δ A at positions 40–57 and 50–65 of GRAB respectively.

Identification of two distinct binding motifs in the Δ A region of GRAB

Based on the results of the initial spot-synthesized peptides, two further sets of membrane-spotted peptides were analysed for α_2 M-binding activity to elucidate the minimal sequences required for binding (Table 2). For this purpose, two overlapping regions, one comprising amino acids 36–61 and the other comprising amino acids 45–70, were each divided into peptides with lengths varying from 8 up to 15 amino acids, with an offset of one amino acid per spot (Figure 5). This strategy resulted in 68 and 63 peptides respectively. The blot-overlay assay with α_2 M, identified 24 strongly reacting spots in the first region and 13 strongly reacting spots in the second region (Figures 5C and 5F). The results suggested an eight-amino-acid peptide, IIPNGGTL, as the minimal binding motif in the first region. Since the N-terminal extension by a proline and an arginine strongly enhanced the α_2 M-binding activity as shown by densitometric analysis, the decapeptide PRIIPNGGTL was proposed as the α_2 M-binding motif of the first region (Figure 5C). The nine-amino-acid peptide NAPEKLALR and the decapeptide NAPEKLALRN were identified as the minimal α_2 M-binding motifs of the second region (Figure 5F). C-terminal extension of the decapeptide with the GRAB-sequence-derived glutamic acid residue resulted in

Figure 5 Analysis of overlapping peptides of different sizes for α_2 M-binding activity

(A, D) Amino acid sequences used for the synthesis of peptides. Binding motifs are underlined. (B) Spot membrane containing eight sets of overlapping peptides with mass ranging from 8 to 15 amino acids, using the initial sequence IIPNGGTL. (E) Spot membrane containing seven sets of synthetic overlapping peptides consisting of 9–15 amino acids each, using NLLGNAPE as the initial sequence. (C, F) Serial numbers and sequences of spots reacting with human α_2 M.

complete loss of α_2 M-binding activity. These results suggested that the two binding motifs contribute substantially to the GRAB- α_2 M interaction.

Systematic mutational analysis by spot-synthesized peptides

From the results of the previous spot membrane analysis, it was predicted that two binding sites of GRAB are involved in the GRAB- α_2 M complex formation. In an attempt to locate the amino acids critical for this interaction, every single amino acid of the peptides PRIIPNGGTL and NAPEKLALRN was individually substituted. Membrane-spotted peptides containing the amino acid substitutions were analysed for their ability to bind soluble α_2 M. Results of the blot-overlay assays revealed that substitutions with glutamic and aspartic acid residues resulted in the complete loss of binding activity in both the binding motifs. In contrast, substitution of each amino acid of the second binding region with a lysine residue resulted in enhanced α_2 M-binding activity. However, the analysis indicated that the arginine residue at position 42 located in the first motif and the arginine residue at position 64 located in the second motif are crucial for the α_2 M-binding activity of GRAB (Figures 6B and 6D). Although substitution of the lysine residue at position 60 resulted in a decrease in the α_2 M binding, this lysine residue was not considered to be crucial for the interaction, since a previous membrane-spotted peptide analysis (Figures 4 and 5F) demonstrated that peptides containing the lysine residue but not the arginine residue (spot 16; Figure 4) are not capable of interacting with α_2 M.

Figure 6 Effects of the α_2 M-binding motifs PRIIPNGGTL and NAPEKLALRN of GRAB using synthetic peptides containing single amino acid substitutions at every position

(A, C) Spot membranes with peptides analysed in an α_2 M ligand binding assay. (B, D) Exchanged amino acids in the motif are underlined. Amino acid substitutions that abolished α_2 M binding are indicated in boldface.

Effect of site-directed mutagenesis of Arg⁴² and Arg⁶⁴ of rGRAB on α_2 M-binding activity

To confirm the critical role of the arginine residues at amino acid positions 42 and 64 of GRAB in α_2 M binding, site-directed mutagenesis was performed. Recombinant GRAB encoded by pQGR was selected for individual amino acid substitutions resulting in rGRAB⁴², rGRAB⁶⁴ and rGRAB^{42/64} (Figure 7A). Binding of α_2 M was first examined using SDS/PAGE (Figure 7B). Results showed that substitution of the arginine residue at position 42 did not affect the binding of α_2 M to rGRAB⁴². In contrast, substitution of the arginine residue at position 64 of GRAB resulted in the complete loss of α_2 M-binding activity under reducing conditions. Binding of α_2 M to rGRAB^{42/64} with amino acid substitutions of both arginine residues was also completely abolished (Figure 7D). Immunoblot analysis using anti-GRAB IgG antibodies was used as a control (Figure 7E). Some other bands in Figures 7(C) and 7(D) are probably GRAB aggregates capable of interacting with α_2 M.

The effect of amino acid substitutions on α_2 M-binding activity was also investigated by competitive inhibition assays (Figure 8). The recombinant proteins rGRAB, rGRAB⁴², rGRAB⁶⁴ and rGRAB^{42/64} were used for competitive inhibition of α_2 M binding to the *S. pyogenes* strain KTL3. Proteins were added separately

Figure 7 Effects of substitutions of the arginine residues in the α_2 M-binding motifs PRIIPNGGTL and NAPEKLALRN on α_2 M binding to recombinant GRAB proteins

(A) Sequences of the recombinant GRAB proteins with individual amino acid substitutions. (B) Coomassie Blue stain of the rGRAB proteins. (C) Immunoblot analysis with rabbit anti-rGRAB IgG. (D) Blot-overlay assay with human α_2 M as the ligand. (E) Immunoblot analysis using rabbit anti- α_2 M IgG alone as a control.

in increasing amounts from 6.6 ng up to 100 μ g per reaction. The competitive inhibition with rGRAB⁴² confirmed the result of the blot-overlay assay that the substitution of R42G did not change the binding of α_2 M to GRAB⁴². Binding of ¹²⁵I- α_2 M to GAS was inhibited by rGRAB⁴² and, therefore, the inhibition was similar to that of the wild-type protein rGRAB. Interestingly, rGRAB⁶⁴, which did not show any α_2 M-binding activity under reducing conditions, could inhibit binding of α_2 M to the bacteria. However, 0.4 nmol (6.25 μ g) of rGRAB⁶⁴ was needed to block nearly 50% of the α_2 M-binding activity of 1.5×10^8 c.f.u. of the *S. pyogenes* strain KTL3, whereas only 0.006 nmol (0.0976 μ g) of wild-type GRAB was sufficient. Substitution of both arginine residues at positions 42 and 64 abolished α_2 M-binding activity under these conditions, as indicated by the lack of inhibiting activity. Therefore these results suggest that Arg⁶⁴ is the most crucial amino acid residue in this interaction, followed by Arg⁴², and both together are crucial for the GRAB- α_2 M complex formation.

DISCUSSION

α_2 M is a plasma protein with a broad spectrum of biological properties. In addition to its well-characterized function as protease inhibitor, α_2 M can interact with cytokines such as IL-1 β (interleukin-1 β), IL-2, IL-6 and IL-8 as well as with growth factors like PDGF- β (platelet-derived growth factor- β), TGF- β (transforming growth factor- β) or NGF- β (nerve growth factor- β), depending on its conformational status (s- α_2 M or f- α_2 M) [39–44]. The ability of α_2 M to interact with all four classes of proteases, irrespective of their origin (exo- or endo-genic), with less efficiency compared with the class-specific inhibitors and the

Figure 8 Competitive inhibition of the binding of ^{125}I - $\alpha_2\text{M}$ to the GAS isolate KTL3

GRAB derivatives were added in increasing concentrations to the $\alpha_2\text{M}$ -binding reaction. (A) Parent rGRAB, (D) rGRAB⁴² (substitution of Arg⁴²), (B) rGRAB⁶⁴ (substitution of Arg⁶⁴) and (C) rGRAB^{42/64} (substitution of Arg⁴² and Arg⁶⁴).

high concentration of $\alpha_2\text{M}$ in human plasma makes $\alpha_2\text{M}$ part of either a protective mechanism decreasing the concentration of soluble plasma proteases to physiological levels or a mechanism against the invading pathogens by presenting entrapped foreign proteases to antigen-presenting cells and therefore stimulating the host immune response [45,46]. The nature of these interactions and its impact on the infection by pathogens remain unclear and open to question. Especially, the ability of $\alpha_2\text{M}$ to interact with a wide spectrum of proteases has been, in turn, used by invading bacteria such as *S. pyogenes* to protect their own surface structures from degradation during the infection process [11,12].

The recruitment of non-protease-complexed s- $\alpha_2\text{M}$ to the GAS surface is mainly mediated by the low-molecular-mass cell-wall-attached protein GRAB. The advantages of this interaction to the bacterial invader are manifold. Binding a broad-range protease inhibitor such as $\alpha_2\text{M}$ provides the bacterium with an important tool to minimize protease degradation of, for example, antiphagocytic surface proteins or to recruit inflammatory mediators to the bacterial surface to enhance local inflammation for a more efficient tissue spreading. Moreover, this mechanism may reduce the concentration of $\alpha_2\text{M}$ in the surrounding environment maintaining protease activity and therefore tissue degradation. The presence of GRAB and its recruitment of $\alpha_2\text{M}$ on to the bacterial surface facilitates bacterial survival and influences the infection process [11]. The fact that GRAB protein contributes to bacterial virulence led to our investigation of the nature of this interaction to predict the structure and stability of the complex or to identify the proteins engaging similar strategies for $\alpha_2\text{M}$ assembly. The present study provides evidence that the recruitment of $\alpha_2\text{M}$ via the surface protein GRAB is mediated by two distinct but adjacent motifs (PR⁴²IIPNGGTL and NAPEKLALR⁶⁴N), which are both located in the ΔA region (amino acids 34–67). Each domain contains a single basic charged arginine residue with distinct roles in the binding facility. Individual substitution of the particular residues influenced the binding in a divergent manner. Substitution of Arg⁴² had no detectable influence on $\alpha_2\text{M}$ -binding activity. In contrast, substitution of Arg⁶⁴ almost resulted in loss of $\alpha_2\text{M}$ -

binding activity of GRAB. Although it was not possible to detect any interaction under reducing conditions, the protein displayed a reduced affinity for $\alpha_2\text{M}$ under physiological conditions. The amino acid substitutions of both residues (Arg⁴² and Arg⁶⁴) emphasized the effect of substitution of Arg⁶⁴. The results obtained strongly indicate that Arg⁶⁴ is the amino acid of GRAB most critical for the interaction with $\alpha_2\text{M}$. As amino acid substitutions of Arg⁴² and Arg⁶⁴ almost completely abolished $\alpha_2\text{M}$ -binding affinity under native conditions, the contribution of the arginine residue at position 42 to complex formation became visible. It is possible that both arginine residues play a role in tertiary-structure formation, assembling the two adjacent motifs in an appropriate position to bind $\alpha_2\text{M}$ with high affinity or just enhance the binding because of their basic charged residues. It is probable that the high affinity of the binding site NAPEKLALR⁶⁴N for $\alpha_2\text{M}$ is masking the contribution of Arg⁴². As a result, binding of $\alpha_2\text{M}$ to rGRAB⁴² is similar to wild-type rGRAB. It is probable, but not confirmed, that the flanking amino acids TLNLL contribute to the high affinity of the GRAB- $\alpha_2\text{M}$ interaction as suggested by spot-membrane analysis. A synthetic peptide IIPNGGTLNLLGNA (Figure 4, spot 10) that did not contain the critical arginine residues was not capable of competitively inhibiting the binding of $\alpha_2\text{M}$ to GAS (results not shown).

Interpretation of these results indicate that the $\alpha_2\text{M}$ -binding activity of GRAB depends on the interplay of the protease inhibitor $\alpha_2\text{M}$ with two adjacent motifs in GRAB. One of these two motifs contains the Arg⁶⁴ and might be directly contributing to the interaction and the other motif, containing Arg⁴², may contribute to high-affinity binding after correct folding of the complex structures or its basic charge. In view of the high sequence similarity between GRAB protein and Protein G, it is tempting to speculate that both proteins have a common ancestor and, accordingly, a closely related mechanism for $\alpha_2\text{M}$ recruitment. The results of the present study indicate the involvement of charged motifs in the ΔA region of GRAB in $\alpha_2\text{M}$ binding and, therefore, in virulence of GAS. These motifs might represent targets for developing new intervention strategies.

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