Functional and structural properties of CbpA, a collagen-binding protein from *Arcanobacterium pyogenes*

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Arcanobacterium pyogenes, an opportunistic pathogen of economically important food animals, is the causative agent of liver abscesses in feedlot cattle, osteomyelitis in turkeys, and pneumonia and arthritis in pigs. Previous studies identified the first *A. pyogenes* adhesin, CbpA, a protein located on the bacterial surface which has the ability to bind collagen and promotes adhesion to the host cells. The protein has an N-terminal ligand-binding region (region A) and a C-terminal repetitive domain (region B). In this study we found that CbpA bound to almost all the collagen types tested but not to other proteins, and it displayed a propensity to interact with several collagenous peptides derived by CNBr cleavage of type I and II collagens. The K_D values of CbpA for type I and II collagens and collagen peptides determined by solid-phase binding assay and intrinsic tryptophan fluorescence were in the range of 1–15 nM. It was also found that CbpA and its A region bound fibronectin, and that collagen and fibronectin interacted with distinct subsites. Anti-CbpA antibodies were effective at inhibiting both binding of isolated CbpA and bacterial adhesion to immobilized collagen, suggesting that CbpA is a functional collagen-binding adhesin. Analysis of the immunological cross-reactivity of CbpA with antibodies against other bacterial collagen-binding proteins indicated that CbpA is immunologically related to ACE from *Enterococcus faecalis* but not to CNA from *Staphylococcus aureus* or Acm from *Enterococcus faecium*. Far-UV and near-UV circular dichroism spectra showed that full-length CbpA and its region A are mainly composed of β -sheet with only a minor α -helical component and that both the proteins have a well-defined tertiary structure.

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INTRODUCTION

Arcanobacterium pyogenes is a ubiquitous inhabitant of the mucous membranes of cattle, swine and many other animal species (Jost & Billington, 2005). As an opportunistic pathogen, *A. pyogenes* can cause a variety of suppurative diseases in animals compromised by previous microbial infection or trauma. Economically significant disease includes liver abscessation in beef cattle, and pneumonia and arthritis in swine (Jost & Billington, 2005). *A. pyogenes* is armed with several factors that contribute to its pathogenesis, including a cholesterol-dependent cytolysin, pyolysin (Billington *et al.*, 1997), several proteases (Schaufuss *et al.*, 1989) and a number of adhesive mechanisms, including two cell-surface neuraminidases (Jost *et al.*,

2002) and a collagen-binding protein, CbpA (Esmay *et al.*, 2003).

Adhesion to the host is an important first step in bacterial colonization and disease pathogenesis. Bacteria target a wide variety of host molecules, but a common mechanism used by a number of pathogens is binding to components of the host extracellular matrix (ECM). In Gram-positive organisms, ECM binding is almost exclusively the property of a number of bacterial surface proteins designated microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Foster & Höök, 1998). CbpA is a 121.9 kDa, surface-expressed protein that binds type I collagen, and is involved in mediating adherence to epithelial and fibroblast cell lines (Esmay *et al.*, 2003). As reported for other MSCRAMMs (Foster & Höök, 1998), CbpA has a modular domain structure consisting of an N-terminal signal peptide, a non-repetitive A region, and four repeated units (B region), followed by a cell-wall anchor region, a transmembrane segment, and a short positively

Abbreviations: CB peptide, collagen fragment generated by CNBr cleavage; CD, circular dichroism; ECM, extracellular matrix; ITF, intrinsic tryptophan fluorescence; MSCRAMM, microbial surface component recognizing adhesive matrix molecules.

charged cytoplasmic tail. In addition, this protein has amino acid similarity to the collagen-binding adhesin CNA of *Staphylococcus aureus*.

A number of studies have directly linked collagen-binding MSCRAMMs to disease processes in collagen-rich tissues. *Staph. aureus* CNA has been demonstrated as a virulence factor in several experimental animal models, including colonization of the joints in mice (Xu *et al.*, 2004a). Similarly, ACE, the collagen adhesin of *Enterococcus faecalis*, is important for adherence to dentin in tooth root canals (Hubble *et al.*, 2003). The critical role of the bacterial attachment to collagen is further indicated by identification of collagen-binding proteins in a variety of micro-organisms such as M3 protein from *Streptococcus pyogenes* (Dinkla *et al.*, 2003), protein FOG in *Streptococcus dysgalactiae equisimilis* (Nitsche *et al.*, 2006), YadA of enteropathogenic *Yersinia* species (Nummelin *et al.*, 2004) and CbsA from *Lactobacillus crispatus* (Antikainen *et al.*, 2002).

CbpA is present in 48 % of *A. pyogenes* isolates; however, 100 % of turkey osteomyelitis isolates were *cbpA*⁺ (Esmay *et al.*, 2003), possibly implicating CbpA in the pathogenesis of osteomyelitis.

This study further extends the biochemical analysis of *A. pyogenes* CbpA with respect to its binding properties for collagens, as well as to structure and immunological correlations with other bacterial collagen-binding proteins.

METHODS

Proteins. Mouse laminin was purchased from BD Biosciences; BSA, ovalbumin and human type III, IV and V collagens were from Sigma. Human fibrinogen (Calbiochem) was made free of contaminating fibronectin by purification over a gelatin-Sepharose column. Fibronectin was purified from human plasma as previously reported (Vuento & Vaheeri, 1979). Recombinant Acn was a generous gift from Dr B. E. Murray (University of Texas Medical School, Houston, TX, USA). CNA19 (residues 151–319), and ACE40 (residues 32–367) were kindly provided by Dr M. Höök (Texas A&M University Health Science Center, Houston, TX, USA).

Collagens and CB peptides. Type I collagen from calf skin was a gift from Dr R. Tenni (Dept of Biochemistry, University of Pavia, Italy). CB peptides were obtained by cleavage of type I collagen with CNBr and purified by gel-filtration chromatography followed by ion-exchange chromatography (Rossi *et al.*, 1996). Type II, IX and XI collagens were purified from bovine nasal septum (Reese & Mayne, 1981). Type II collagen CNBr peptides were isolated essentially following the procedures used for peptides from type I collagen (Rossi *et al.*, 1996). All collagens and peptides were analysed for purity by a quantitative Hyp assay (Huszar *et al.*, 1980), by electrophoresis in denaturing conditions (Laemmli, 1970), and by N-terminal sequencing for some peptides, and for conformation by means of circular dichroism spectroscopy.

Expression and purification of CbpA proteins. Cloning of recombinant CbpA, lacking the signal peptide (residues 28–1151), and the A domain of CbpA (residues 29–355) was performed as reported previously (Esmay *et al.*, 2003).

The fusion proteins, containing an N-terminal His-tag, were purified by immobilized metal chelate affinity chromatography and gel filtration chromatography on a Superose 12 HR column fitted on an FPLC system. The purity of the isolated proteins was assessed by SDS-PAGE.

Bacteria and growth conditions. *Escherichia coli* DH5 α MCR harbouring plasmid pJGS591 (Esmay *et al.*, 2003) was routinely grown in Luria broth or in Luria agar (Difco) containing 100 μ g ampicillin ml⁻¹ at 37 °C. *Enterococcus faecium* 395 cultures were grown in Brain Heart Infusion (BHI; Difco) at 37 °C. *Enterococcus faecalis* 706897 was grown in BHI medium at 46 °C. *A. pyogenes* BBR1 was grown in BHI supplemented with 5 % (w/v) newborn calf serum and 5 μ g tetracycline ml⁻¹ at 37 °C.

Biotin labelling of CbpA. CbpA (0.5 mg) dissolved in 0.5 ml PBS, pH 7.4, was mixed with 0.5 ml 0.2 M sodium borate buffer, pH 8.0, and 7.5 mg *N*-hydroxysuccinimidobiotin (NHS-biotin) (Sigma) dissolved in 0.1 ml DMSO, and incubated at 22 °C overnight with end-over-end mixing. The labelling mixture was dialysed against PBS and stored at –20 °C in small aliquots.

Polyclonal and monoclonal antibodies. Antibodies to recombinant CbpA, ACE40, CNA19 and Acn were raised in BALB/c mice. Injections were given intraperitoneally four times at 1 week intervals with 50 μ g of the antigen emulsified with an equal volume of complete Freund's adjuvant for the first immunization, followed by three injections in Freund's incomplete adjuvant. The mice were bled and the sera were tested for reactivity against the corresponding antigen.

The mAbs against ACE40 and CNA19 were produced essentially as described by Köhler & Milstein (1975) with minor modifications. Hybridoma supernatants were screened for reactivity with the appropriate antigen immobilized on microtitre plates and positive clones were further characterized by ELISA and Western blotting.

Antibodies from the sera and hybridoma supernatants were purified by affinity chromatography on protein G-Sepharose according to the recommendations of the manufacturer (Amersham Biosciences). Isotyping of the mAbs produced was performed using a Mouse-Typer subtyping kit (Bio-Rad). All the mAbs were found to belong to the IgG isotype.

ELISA. The binding of CbpA proteins to plasma or ECM proteins was determined in an ELISA-type assay. Unless otherwise stated, microtitre wells were coated with 100 μ l of 10 μ g ml⁻¹ of the indicated proteins dissolved in 50 mM sodium carbonate, pH 9.5. To block additional protein-binding sites, the wells were treated for 1 h with 200 μ l 2 % (w/v) BSA in PBS at 22 °C. The wells were then washed with PBST (PBS+0.5 %, v/v, Tween 20) and incubated for 1 h with 1 μ g CbpA or region A of CbpA. After washing, bound ligand was incubated for 1 h with 1 μ g mouse anti-CbpA IgG followed by 1 h incubation with peroxidase-conjugated rabbit anti-mouse IgG (Dako). The conjugated enzyme was allowed to react with *o*-phenyldiamine dihydrochloride (Sigma) and absorbance at 490 nm was measured using a microplate reader (Bio-Rad). To calculate the relative association constant (K_a) values of CbpA proteins for type I and II collagens and collagen peptides from the saturation kinetics, the following equation was used: $A = A_{\max}[\text{CbpA}]/(1 + K_a[\text{CbpA}])$.

The reported K_D values were calculated as the reciprocal of the corresponding association constants.

To measure the adsorption of each protein to the plates, protein bound to the wells was determined by using a BCA protein assay kit, Microplate Procedure, Protocol #2161297A (Pierce): in these conditions, a 80–90 % coating efficiency was detected for each protein tested.

The binding of CbpA to collagen in the presence of anti-CbpA antibodies was studied by incubating collagen-coated microtitre plates (1 µg per well) with 1 µg biotin-labelled CbpA for 1 h at 22 °C. CbpA bound to collagen was detected by the addition of a 1:2000 dilution of avidin-peroxidase followed by a chromogenic substrate, and absorbance at 490 nm was measured using a microplate reader.

Attachment of *A. pyogenes* cells to collagen-coated wells was performed by incubating microtitre plates coated with collagen type I (1 µg per well) with 5×10^7 bacterial cells for 90 min. After three washes, collagen-bound bacteria were incubated with 1 µg of a rabbit anti-*A. pyogenes* IgG. Binding of antibody to bacteria was detected by addition of a peroxidase-labelled goat anti-rabbit secondary antibody.

Immunological reactivity of collagen-binding proteins. Cross-reactivity of recombinant adhesins from *A. pyogenes* (CbpA), *Staph. aureus* (CNA), *Ent. faecalis* (ACE) and *Ent. faecium* (Acm) was analysed by incubating adhesin-coated microtitre wells (1 µg per well) with 1 µg mouse IgG isolated from immune sera against each adhesin or with 1 µg of individual mouse monoclonal antibodies raised against ACE40. Binding of the antibodies to the wells was detected by addition of 1 µg peroxidase-conjugated rabbit anti-mouse antibody.

Immunological reactivity of bacterial species expressing collagen-binding proteins. Microtitre wells were coated with 100 µl of bacterial cells (5×10^7 per well) in PBS, overnight at 37 °C. After incubating with 2% (w/v) BSA the bacteria-coated wells were washed with PBST and incubated for 90 min with 1 µg IgG of individual monoclonal antibodies against CNA19 or ACE40. The wells were washed three times and incubated with 1:1000 dilution of peroxidase-conjugated rabbit anti-mouse antibody. The conjugated enzyme was allowed to react with *o*-phenyldiamine dihydrochloride and absorbance at 490 nm was measured using a microplate reader.

Circular dichroism (CD) spectroscopy. Far-UV (195–250 nm) and near-UV (250–320 nm) CD measurements were performed at 20 °C in 0.1 and 1.0 cm pathlength quartz cuvettes, respectively. CD spectra were recorded on a Jasco J-720 spectropolarimeter. The results are expressed as the mean residue ellipticity assuming a mean residue weight of 110 amino acid residues. All the spectroscopic measurements were performed in 20 mM phosphate buffer pH 7.4. Six scans were averaged for each spectrum, and the contribution from the buffer was subtracted in each case. Quantification of secondary structural components was performed using the deconvolution programs CONTIN, CDSSTR and SELCON3.

Intrinsic tryptophan fluorescence. The intrinsic tryptophan fluorescence of 0.2 µM CbpA proteins in PBS was examined with a Jasco FP-6500 spectrofluorimeter at 22 °C. The excitation wavelength was set at 295 nm (5 nm slit width) while monitoring emission from 305 to 400 nm (5 nm slit width). Quenching of tryptophan fluorescence after the addition of collagen (from 0.0015 to 0.1 µM) was analysed via a modified Stern–Volmer plot (Eftink & Ghiron, 1981) as follows. The $F_0/(F_0 - F)$ ratio (where F_0 and F are the fluorescence intensities at 338 nm in the absence and presence of collagen, respectively) plotted against the reciprocal of CbpA concentration yields a straight line, the intercept of which on the x axis equals the value of the association constant (K_a) for collagen.

RESULTS

Specific binding of CbpA to collagens

CbpA was previously found to mediate binding of *A. pyogenes* to collagen (Deivanayagam *et al.*, 2000). To

analyse the specificity of interactions of CbpA with collagen, we immobilized a number of ECM or plasma proteins onto microtitre wells and then incubated the plates with recombinant full-length CbpA or region A of CbpA. Binding of recombinant CbpAs to immobilized proteins was detected by addition of an anti-CbpA antibody to the wells. In confirmation of previous data, CbpA proteins bound to type I collagen but not to other ligands such as laminin, elastin, ovalbumin or albumin (Fig. 1a). Interestingly, CbpA and its A domain were found to interact also with fibronectin and to a lesser extent with fibrinogen. The ability of CbpA to bind both collagen and fibronectin appeared to be a peculiar property of CbpA, because other bacterial collagen-binding proteins such as CNA from *Staph. aureus* and ACE from *Ent. faecalis* did not bind to fibronectin (data not shown). With exclusion of collagen V and IX, for which CbpA showed reduced binding, the binding of CbpA to genetically distinct collagen types was very similar (Fig. 1b).

To further demonstrate the specific interaction of CbpA with collagen, a competitive binding assay was set up in which CbpA was pre-incubated with soluble type I collagen or the proteins fibronectin, fibrinogen and ovalbumin, and then tested for binding to surface-coated type I collagen. Pre-incubation of CbpA with increasing amounts of soluble collagen resulted in inhibition of the protein binding to immobilized collagen by 80% at the highest concentration of soluble competitor, while a negligible effect was detected when CbpA was pre-incubated with fibronectin, fibrinogen or ovalbumin (Fig. 1c).

Saturable binding of CbpA to collagens and CB peptides

To provide further evidence that CbpA was a relevant partner for binding to collagen, microtitre wells coated with type I collagen were incubated with increasing amounts of CbpA or region A of CbpA. As shown in Fig. 2, full-length CbpA and region A bound to collagens in a dose-dependent fashion, whereas the binding to the wells coated with albumin was minimal (data not shown). From these assays we estimated the K_D value of full-length CbpA and region A for type I collagen as 5.26 and 1.12 nM, respectively (Table 1). K_D values in the same range were obtained by incubating increasing concentrations of CbpA or region A of CbpA with surface-coated type II collagen (Table 1).

The full-length CbpA and region A contain 20 and 4 tryptophan residues, respectively, whereas no tryptophan residues are present in the triple helix of type I or II collagen. The fluorescence spectra of CbpA and of region A showed fluorescence emission maxima at 340 and 350 nm, respectively; the difference in quantum yield for region A is presumably caused by the lower number of tryptophan residues (data not shown).

Preliminary analysis indicated that the binding of type I or II collagen to CbpA resulted in quenching of intrinsic

Fig. 1. (a) CbpA specifically binds to collagens: CbpA binding to surface-coated type I collagen and extracellular or plasma proteins. The indicated proteins (1 μ g) were immobilized overnight onto microtitre wells. Plates were incubated with 1 μ g full-length CbpA or region A of CbpA for 60 min at 22 °C, and after washing, 50 ng mouse anti-CbpA IgG was added for 90 min. Antibody binding was determined by incubating the wells with 1 : 1000 dilution of a peroxidase-conjugated rabbit anti-mouse antibody. (b) Binding of CbpA to different collagen types. Microtitre wells coated with 1 μ g of the indicated collagens were incubated with 1 μ g CbpA for 60 min. The binding of CbpA to the immobilized substrate was detected as reported above. (c) Dose-dependent effect of soluble type I collagen, fibronectin, fibrinogen and ovalbumin on the binding of CbpA to immobilized type I collagen. Binding of CbpA to collagen was detected as reported in (a). Data are expressed as a percentage of CbpA binding in the absence of competitors. Values represent the means $\pm$ SD of triplicate wells.

Fig. 2. CbpA binds type I collagen in a dose-dependent and saturable manner. Microtitre wells were coated with 1 μ g collagen I and then analysed for the binding of increasing amounts of full-length CbpA or region A of CbpA. Binding of the ligand was detected as reported in Fig. 1(a). Values represent the means $\pm$ SD (error bars not shown where smaller than symbols) of triplicate wells. This experiment was repeated three times with similar results.

tryptophan fluorescence (ITF). We therefore measured the fluorescence of CbpA in the presence of increasing concentrations of collagen I or II when the protein was excited at 295 nm (Fig. 3a, b). Collagens quenched the intrinsic fluorescence of CbpA in a dose-dependent manner, whereas no quenching of fluorescence emission was detected when only PBS or non-collagenous proteins were added to the mixtures of the recombinant proteins. Analysis of the tryptophan quenching data by a modified Stern–Volmer plot gave K_D values of 2.23 and 1.0 nM, respectively, for collagens I and II (Fig. 3a, b, Table 1). Affinities of region A for type I and II collagens as measured by ITF were 2.27 and 6.86 nM, respectively (Table 1).

To identify CbpA binding sites in collagen, we examined the binding of CbpA to fragments of type I or II collagen generated by cyanogen bromide cleavage (CB peptides). CbpA bound almost all the fragments tested (Fig. 4) although a preferential binding of CbpA to the peptides CB3.8(I), CB7(I), CB8(I) and CB11(II) was observed. However, it remains to be determined whether the better recognition of these peptides by CbpA reflects the interaction with specific binding sites or is the result of a differential coating efficiency when peptides of different size are tested.

Saturation kinetics of CbpA or region A of CbpA for CB peptides CB3.8(I), CB8(I) and CB11(II) determined either by solid-phase binding assay or by ITF measurements gave K_D values in the range of 1–16 nM (Table 1).

Anti-CbpA antibodies block interaction of CbpA with collagen

Purified IgG antibodies isolated from an antiserum raised against full-length CbpA were tested for their ability to

Table 1. K_D values (nM) of CbpA protein for collagen and CB peptides

	Collagen type I		Collagen type II		CB3.8 (I)		CB8 (I)		CB11 (II)	
	ELISA	ITF	ELISA	ITF	ELISA	ITF	ELISA	ITF	ELISA	ITF
CbpA	5.26	2.23	15	1.0	14	1.64	14.2	1.07	16	1.66
A domain	1.12	2.27	3.73	6.86	6.25	3.14	7.57	5.22	8	3.33

inhibit CbpA-dependent *A. pyogenes* adherence to a collagen substrate. Anti-CbpA IgG was a potent inhibitor of cell adhesion, causing 85 % inhibition at 10 $\mu\text{g ml}^{-1}$

(Fig. 5). A similar blocking effect was obtained when collagen-coated wells were incubated with biotin-labelled CbpA in the presence of increasing concentrations of the

Fig. 3. Analysis of CbpA binding to type I (a) and type II (b) collagens by fluorescence quenching. Top panels, tryptophan fluorescence emission spectra of full-length CbpA in the absence or presence of increasing amounts of type I or II collagen. No quenching was observed when CbpA was incubated with PBS or non-collagenous proteins. Middle panels, quenching of CbpA intrinsic fluorescence in the presence of increasing concentrations of collagen. The intrinsic fluorescence of CbpA in the absence of collagen was set as the relative fluorescence intensity equal to 100. Bottom panels, modified Stern–Volmer plots of the data acquired from the fluorescence quenching assay.

Fig. 4. Binding of CbpA to CB peptides. Microtitre wells coated with 1 µg of the indicated CB peptides were incubated with 1 µg CbpA for 60 min. The binding of CbpA to the immobilized substrate was detected as reported in Fig. 1(a). The data are means ± SD of triplicate samples.

same antibody (data not shown). Conversely, no effect was detected when either bacteria or the recombinant CbpA were incubated with immobilized collagen in the presence of IgG isolated from a pre-immune serum.

Immunological cross-reactivity of antibodies against collagen-binding proteins from different bacterial species

CbpA, CNA from *Staph. aureus*, ACE from *Ent. faecalis* and Acm from *Ent. faecium* are collagen-binding proteins exhibiting significant residue similarity (Esmay *et al.*, 2003). Thus, it was of interest to analyse the possible immunological cross-reactivity of these adhesins. To this end, CbpA was investigated in ELISA format for the reactivity with mAbs against the N2 module of region A of CNA (CNA19) (Visai *et al.*, 2000) or ACE40, which are recombinant segments encompassing the collagen-binding site in these two adhesins (Patti *et al.*, 1993; Rich *et al.*, 1999). mAbs against CNA strongly reacted with the immobilized CNA19 fragment, but did not recognize immobilized CbpA to any degree (data not shown). The collection of mAbs raised against ACE40 reacted well with homologous antigen, and a number of mAbs (3E11, 8A1, 9D4, 10E4, 11A6, 11C7, 11D12 and 12A2) also recognized CbpA (Fig. 6a). However, the relative affinity of these antibodies for CbpA epitopes was reduced ~100-fold compared to that for ACE homologous epitopes (K_D values of 100 nM vs 1 nM, respectively). To analyse whether similar cross-reactivity would be observed under more physiological conditions, the panels of mAbs against CNA and ACE were examined for reactivity with adhesins directly expressed on the surface of bacteria. Consistent with the poor cross-reactivity of the anti-CNA monoclonal antibodies with recombinant CbpA, the mAbs against CNA

Fig. 5. Antibodies against CbpA block the interaction of CbpA with collagen. Surface-coated collagen type I was incubated for 90 min at 37 °C with 5×10^7 cells of *A. pyogenes* in the presence of increasing concentrations of mouse anti-CbpA IgG. After extensive washing, collagen-bound bacteria were incubated with 1 µg of a rabbit anti-*A. pyogenes* IgG. Binding of antibody to bacteria was detected by addition of a peroxidase-labelled goat anti-rabbit secondary antibody. The values are expressed as percentages of the control lacking inhibitor. The effect of preimmune IgG on adherence of bacteria to collagen is shown as a control. Bars represent SD from the means of triplicate determinations.

did not show any reactivity towards CbpA expressed on the surface of *A. pyogenes* (data not shown). Conversely, an even wider reactivity was noticed following incubation of the panel of anti-ACE mAbs with immobilized cells of *A. pyogenes*, indicating a more favourable display of the epitopes when CbpA was presented on the bacterial cell surface (Fig. 6b). Similar results were obtained when CbpA or *A. pyogenes* cells were reacted with polyclonal antibodies against CNA or ACE40. A lack of cross-reactivity was observed when recombinant CbpA or *A. pyogenes* cells were incubated with polyclonal antibodies against Acm (data not shown).

Spectroscopic characterization of CbpA

The calculated molecular masses (from the primary amino acid sequence) of the full-length CbpA and region A were 121.9 kDa and 35.8 kDa, respectively. CbpA and region A eluted from a size-exclusion chromatography column (Superose 12 HR 10/30) at a position equivalent to 163 kDa and 24 kDa, respectively (data not shown). This result suggests that full-length CbpA may adopt an extended conformation, whereas region A appears smaller than expected and more compact.

The spectral properties of full-length CbpA and of region A were investigated by CD. Far-UV CD spectra of the full-length CbpA and the A domain showed a single maximum and minimum at 200 and 215–220 nm, respectively (Fig. 7a). Deconvolution of the CD spectra by using three independent procedures showed that the proteins are

Fig. 6. Immunological cross-reactivity of recombinant collagen-binding proteins CbpA and ACE. (a) Collagen-binding proteins CbpA and ACE40 (each 1 µg) were immobilized onto microtitre wells. Plates were incubated with 1 µg mouse monoclonal antibodies against ACE40 and bound antibody detected by addition of a peroxidase-conjugated rabbit anti-mouse antibody. (b) *A. pyogenes* BBR1 or *Ent. faecalis* 706897 (5×10^7 cells each) immobilized onto microtitre wells were incubated with 1 µg mAbs against ACE40. Binding of antibody to cells was detected as described for Fig. 1(a). Data are means $\pm$ SD of triplicate wells.

mainly composed of β -sheets and indicated the relative content of secondary structure elements of both the proteins (Table 2). The near-UV CD spectra of CbpA and of region A indicated that both proteins are folded into a compact tertiary structure (Fig. 7b) and the differences in dichroic activity for region A may be due to the lower

number of aromatic residues. All the aromatic residues contribute to the near-UV CD spectra of CbpA and region A: signals around the region 260–270 nm are attributable to phenylalanine residues, signals from 275 to 288 nm are attributable to tyrosine and those from 290 to 300 nm to tryptophan. The near-UV CD spectrum of CbpA exhibited

Fig. 7. Spectroscopic analysis of CbpA. Far-UV CD (a) and near-UV CD (b) spectra of CbpA and region A were recorded at 20 °C in 20 mM phosphate buffer at pH 7.0. Far-UV CD spectra were recorded at 0.1 mg ml⁻¹ protein concentration. The predicted secondary structure composition of each protein is reported in Table 2. Near-UV CD spectra were recorded in a 1 cm quartz cuvette at 0.90 and at 1.28 mg ml⁻¹ protein concentration for CbpA and region A, respectively. The reported spectra are the average of five scans, corrected for buffer blank, and were smoothed.

maxima at 278, 285, 290 and 294 nm. The near-UV CD spectrum of region A showed a positive band in the tryptophan region but differed at shorter wavelengths from those of full-length CbpA. In conclusion, the spectral profiles of both the proteins suggested that they have a well-defined tertiary structure and that all the aromatic residues are locked into tertiary contacts.

DISCUSSION

A family of structurally related collagen-binding proteins is found on Gram-positive bacteria; among them are CNA

from *Staph. aureus* (Patti *et al.*, 1992), ACE from *Ent. faecalis* (Nallapareddy *et al.*, 2000) and Acm from *Ent. faecium* (Nallapareddy *et al.*, 2003). We previously identified the first *A. pyogenes* MSCRAMM, CbpA, which is capable of binding type I collagen (Esmay *et al.*, 2003). In this study we further examined the binding properties of CbpA to collagens, compared the immunological cross-reactivity of CbpA with other bacterial collagen-binding adhesins and analysed some of the structural aspects of the protein. First, we demonstrated that CbpA and the A region of CbpA bind to type I and II collagen specifically, saturably and with similar affinities. The apparent K_D values of the interaction between type I or II collagen and CbpA proteins are in the range of 1–15 nM, as determined by ELISA and ITF; these values are lower than those observed for the collagen-binding proteins rBA0871A and rBA5258A from *Bacillus anthracis* (Xu *et al.*, 2004b) and ACE from *Ent. faecalis* (Rich *et al.*, 1999).

An interesting question is whether CbpA behaves promiscuously in its binding to different collagen types. Our data indicate that this is the case. In fact, CbpA recognizes almost all the collagen types tested. This feature could broaden the spectrum of potential *A. pyogenes* infection sites and make this bacterium less dependent on local variation in tissue composition and availability of specific components, which is consistent with the ability of this organism to infect a variety of host species and tissue types (Jost & Billington, 2005). In addition, CbpA binds a number of peptides generated by CNBr cleavage of type I and II collagens.

The binding of CbpA to different CB peptides is consistent with the presence of highly repetitive sequences in the collagen α -chains. This suggests that the CbpA binding sites are scattered over the collagen molecule and that CbpA can bind many sites along the collagen molecule. In addition, on the basis of the tendency of CB peptides to form homologous homotrimers (Rossi *et al.*, 1996) and the similar affinity of CbpA for both collagens and CB peptides we can speculate that collagen triple helix is the recognition element for CbpA binding. Recently, several bacterial surface proteins that were regarded as binding a single ligand were shown to bind additional host proteins. For example, the fibronectin-binding proteins FnbpA and FnbpB from *Staph. aureus* also recognize fibrinogen and elastin (Roche *et al.*, 2004), while ClfB, originally described as a fibrinogen-binding staphylococcal receptor, binds to

Table 2. Summary of secondary structural components in CbpA protein

Values reported here are an average of results obtained using the CONTIN, CDSSTR and SELCON3 deconvolution programs.

	α -Helix	β -Sheet	Turns	Random
CbpA	0.042 $\pm$ 0.03	0.373 $\pm$ 0.05	0.229 $\pm$ 0.032	0.323 $\pm$ 0.007
A domain	0.044 $\pm$ 0.014	0.42 $\pm$ 0.009	0.216 $\pm$ 0.008	0.316 $\pm$ 0.017

cytokeratin 10 (Walsh *et al.*, 2004). In line with this, here we show a second binding activity for CbpA. Both recombinant full-length CbpA and the A region of CbpA bind fibronectin. In addition, we tested the competitive effect of type I collagen on CbpA binding to surface-coated fibronectin or, conversely, the potential interference of soluble fibronectin on the binding of CbpA to immobilized type I collagen in ELISA assays. In these conditions collagen did not affect CbpA binding to fibronectin, nor did fibronectin influence CbpA binding to collagen (data not shown and Fig. 1c). These findings rule out the possibility that binding of CbpA to either ligand could be attributed to an artefact of collagen or fibronectin contamination. Moreover, the absence of cross-inhibition by one ligand on the binding of the other one to CbpA clearly indicates that collagen and fibronectin recognize different subsites of CbpA.

Vaccination of animals with a recombinant form of CNA or RspA, the collagen adhesins of *Staph. aureus* and *Erysipelothrix rhusiopathiae*, was shown to protect against challenge by wild-type *Staph. aureus* (Nilsson *et al.*, 1998) and *E. rhusiopathiae* (Shimoji *et al.*, 2003), respectively. The finding that CbpA is a good immunogen and that antibodies against CbpA inhibit *A. pyogenes* attachment to immobilized collagen suggests that CbpA might be a vaccine candidate for combating infections by this bacterium.

It has been shown that region A of CbpA exhibits similarity with A regions of other collagen-binding proteins, with the most similarity to the A region of CNA (percentage sequence identity and similarity 28% and 68%, respectively). Surprisingly, we found that the immunological cross-reactivity between CbpA and CNA is very limited, as suggested by the weak recognition by the panel of mAbs against CNA of either isolated, recombinant or *A. pyogenes* surface-exposed CbpA. In contrast, in spite of the relatively lower sequence similarity between the A domains of CbpA and ACE (24% identity, 54% similarity), a number of anti-ACE monoclonal antibodies recognize CbpA in an ELISA. However, the affinity of these anti-ACE mAbs is 100-fold lower than that for the homologous epitopes. An even wider response is observed by incubating *A. pyogenes* cells with anti-ACE mAbs, indicating that some epitopes that are exposed on the bacterial surface are more accessible than the corresponding ones present in the recombinant protein immobilized onto microtitre wells.

It has been shown that a recombinant form of A region of CNA (Zong *et al.*, 2005) or ACE (Rich *et al.*, 1999) has a β -sheet structure that folds into an Ig-like fold. Our CD analysis indicates that CbpA and the A region of CbpA are composed primarily of β -sheet structures. For this reason and on the basis of the analysis of the three-dimensional structure of A region by homology modelling (data not shown), it is plausible that the CbpA folds similarly to ACE and CNA and adopts an Ig-like fold. In addition, the finding that full-length CbpA and its A region have a substantially similar percentage of β -sheet structure

suggests that, consistent with the structure of the repetitive units of CNA (Deivanayagam *et al.*, 2000), the B domains of CbpA might have a β -sheet arrangement.

Earlier studies indicated that the B domains of CNA neither bind collagen nor influence the A region's collagen-binding activity (Rich *et al.*, 1998). These B region repeat units have been proposed to serve as a stalk that projects the A region away from the staphylococcal cell surface and positions it for binding to collagen. Thus, it is reasonable to suppose that the B domain in CbpA has a similar function. In conclusion, as previously reported for CNA and ACE, CbpA is composed primarily of β -sheet structures, with a minor α -helical component, but the global arrangement of such secondary structural elements in this MSCRAMM remains to be determined.

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