The Human Polymeric Immunoglobulin Receptor Binds to
*Streptococcus pneumoniae* via Domains 3 and 4*

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**Ling Lu‡, Michael E. Lamm§, Hongmin Li¶, Blaise Corthesy‡, and Jing-Ren Zhang**

*From the ‡Center for Immunology and Microbial Disease, Albany Medical College, Albany, New York 12208, the §Department of Pathology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106, the Wadsworth Center, New York State Department of Health, Albany, New York 12201, and the ¶Division of Immunology and Allergy, R & D Laboratory, Centre Hospitalier Universitaire Vaudois, 1005 Lausanne, Switzerland*

*Streptococcus pneumoniae* (the pneumococcus) is a major cause of bacterial pneumonia, middle ear infection (otitis media), sepsis, and meningitis. Our previous study demonstrated that the choline-binding protein A (CbpA) of *S. pneumoniae* binds to the human polymeric immunoglobulin receptor (pIgR) and enhances pneumococcal adherence to and invasion of cultured epithelial cells. In this study, we sought to determine the CbpA-binding motif on pIgR by deletional analysis. The extracellular portion of pIgR consists of five Ig-like domains (D1–D5), each of which contains 104–114 amino acids and two disulfide bonds. Deletional analysis of human pIgR revealed that the lack of either D3 or D4 resulted in the loss of CbpA binding, whereas complete deletions of domains D1, D2, and D5 had undetectable impacts. Subsequent analysis showed that domains D3 and D4 together were necessary and sufficient for the ligand-binding activity. Furthermore, CbpA binding of pIgR did not appear to require Ca**2** or Mg**2**. Finally, treating pIgR with a reducing agent abolished CbpA binding, suggesting that disulfide bonding is required for the formation of CbpA-binding motif(s). These results strongly suggest a conformational CbpA-binding motif(s) in the D3/D4 region of human pIgR, which is functionally separated from the IgA-binding site(s).

This explains why virtually all virulent strains express the capsule (2). Pneumococcal surface protein A (PspA)**1** is a surface-exposed protein virulence factor. It has been shown to enhance pneumococcal survival in mice by interfering with recruitment of the alternative complement pathway (4, 5). Pneumolysin, a major pneumococcal toxin, is able to deplete complement by activating the classical complement pathway (6, 7) and inhibiting bactericidal activity of neutrophils (8).

Several surface-associated molecules of *S. pneumoniae* have been identified as contributing to pneumococcal adhesion to respiratory epithelium. The cell wall phosphorylcholine binds to receptor for the platelet-activating factor, which adheres pneumococci to lung epithelial cells (9). Pneumococcal surface adhesin A (PsaA), a component of the manganese transport system in *S. pneumoniae* (10, 11), has been shown to enhance pneumococcal adhesion to human nasopharyngeal epithelial cells (12). Consistently, pneumococcal strains lacking PsaA exhibit reduced nasopharyngeal colonization and virulence in mice (13). A fibronectin-binding protein of *S. pneumoniae* designated pneumococcal adhesin/virulence protein A has been shown to play a role in adhesion and virulence in mice (14). Choline binding protein A (CbpA), a cell surface-exposed protein of *S. pneumoniae*, is structurally related to PspA (15). Mutagenesis studies have demonstrated that CbpA is required for pneumococcal nasal colonization (16, 17) and lung infection (16, 18, 19).

CbpA, also known as PspC (20), or SpaA (21), belongs to a family of proteins that are tethered to the pneumococcal cell surface by a choline-binding domain at the C termini (17, 22). In serotype 2, mature CbpA consists of 663 amino acids with a predicted mass of 75 kDa (17). CbpA has been shown to bind to multiple host factors. Hammerschmidt et al. have shown that SpA, a CbpA variant, binds human free secretory component (SC) and human secretory immunoglobulin A (S-IgA) via a hexapeptide motif (YRNYPY) (21). CbpA has also been shown to bind to complement proteins C3 (23) and factor H (24, 25). CbpA binding to C3 facilitates pneumococcal adhesion to human lung epithelial cells (26). Binding to factor H by a CbpA homolog in type 3 pneumococci significantly inhibits complement activation and phagocytosis in vitro (27). Furthermore, CbpA has been shown to stimulate cytokine production by cultured lung epithelial cells (28, 29). These studies suggest

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**The abbreviations used are:** PspA, pneumococcal surface protein A; PsaA, pneumococcal surface adhesin A; BSA, bovine serum albumin; CbpA, choline-binding protein A; CMV, cytomegalovirus; DTT, 1,4-dithiothreitol; ELISA, enzyme-linked immunosorbent assay; MDCK, Madin-Darby canine kidney; pIgR, polymeric immunoglobulin receptor; PBS, phosphate-buffered saline; PspC, pneumococcal surface protein C; SC, secretory component; S-IgA, secretory immunoglobulin A; SpaA, *S. pneumoniae* SC/S-IgA binding protein A.

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**To whom correspondence should be addressed: Center for Immunology and Microbial Disease, Albany Medical College, M/C 151, Albany, NY 12208. Tel.: 518-262-6412; Fax: 518-262-6161; E-mail: zhang@mail.alma.edu.
that CbpA acts as a multifunctional virulence factor during pneumococcal infection in the host. Our previous study demonstrated that CbpA binds the epithelial polymeric immunoglobulin receptor (pIgR) through interaction with its SC region, i.e. the external portion of pIgR (30). CbpA-pIgR interaction significantly enhances pneumococcal adhesion to human nasopharyngeal epithelial cells and Madin-Darby canine kidney (MDCK) cells transfected with the human pIgR cDNA. Moreover, CbpA interaction with pIgR increases the level of pneumococcal invasion by 10- to 50-fold in pIgR-expressing epithelial cells (30). This finding suggests that CbpA-pIgR interaction promotes pneumococcal infection in vivo.

pIgR is broadly expressed by mucosal epithelium to transport polymeric immunoglobulins (IgA and IgM) across the mucosal epithelial barrier (31). Synthesized IgA and IgM in subepithelial tissues bind to pIgR at the basolateral surface of the epithelium and are transported to the apical surface, where the extracellular region of pIgR, also called SC, is proteolytically cleaved to release IgA or IgM with bound SC in mucosal secretions. The SC-containing IgA is referred to as S-IgA. S-IgA is the predominant antibody isotype in mucosal secretions. The main biological function of S-IgA is to prevent pathogenic bacteria from colonizing and invading mucosal epithelia (32). Free SC is also naturally present in mucosal secretions, but its biological function is not completely clear. Free SC is thought to enhance innate immune responses by fixing the complement component C3b (33), inducing eosinophil degranulation (34), and binding to pathogenic bacteria (35) and bacterial toxins (36).

The human pIgR protein is composed of 764 amino acids with a calculated molecular size of 81.5 kDa. It is a type I transmembrane protein of the immunoglobulin (Ig) superfamily with five Ig-like extracellular domains (104–114 amino acids for each domain), a transmembrane segment (23 amino acids), and a cytoplasmic domain (103 amino acids) (37). The N-terminal domain 1 has been shown to bind to the Cα3 loop of dimeric IgA (38–40) and the J chain (41). Crottet et al. (42) have shown that domains 2 and 3 of murine SC are not necessary for covalent binding to IgA. Human pIgR shares various degrees of amino acid sequence identities with other pIgRs necessary for covalent binding to IgA. Human pIgR shares various degrees of amino acid sequence identities with other pIgRs such as rabbit (55%), rat (65%), mouse (66%), and bovine (67%). Previous studies have localized the CbpA-binding activity to the extracellular SC portion of human pIgR (30, 43). Although pIgR is highly N-glycosylated (44–46), treatment of human SC with sodium periodate did not alter CbpA-binding activity (30), suggesting that carbohydrate on human pIgR is not necessary for CbpA binding. The objective of this study was to localize the CbpA-binding motif on human pIgR.

EXPERIMENTAL PROCEDURES

Cell Cultures and Molecular Biology Reagents—MDCK strain II and COS-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 0.1 mM non-essential amino acids and 10% (v/v) fetal calf serum (Invitrogen). Cells were maintained in a 5% CO2 incubator at 37 °C. All restriction enzymes and DNA markers were obtained from New England Biolabs (Beverly, MA). Mammalian protein expression vector pcDNA3.1+ (−) was purchased from Invitrogen. EDTA, EGTA, 1,4-dithiothreitol (DTT), and isoaoxacamide were obtained from Sigma (St. Louis, MO). Anti-CbpA (30) or anti-human IgA, respectively, purified from human milk (48) and the serum of a single myeloma patient by precipitation in ammonium sulfate and fast protein liquid chromatography fractionation. Purified recombinant murine SC was constructed and expressed in mammalian cells as described previously (42). A recombinant form of CbpA, CbpA2, was expressed and purified as a six-histidine-tagged protein in Escherichia coli as described previously (30).

For immunoblotting analysis, proteins or mammalian cell lysates were boiled in the presence of 1% β-mercaptoethanol, subjected to 10% SDS-PAGE separation, and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by using a semi-dry transfer system (Bio-Rad, Hercules, CA). Protein blots were reacted with primary antibodies diluted 1:2,000–5,000 in phosphate-buffered saline (PBS). Bound antibody was detected by reaction with appropriate secondary antibody-peroxidase conjugate (1:10,000 dilution) using an enhanced chemiluminescence (ECL) Western blot kit (Amersham according to the supplier’s instructions). Immunofluorescence staining of mammalian cells was performed according to standard protocols (50), and detections were carried out with an Olympus BX51 fluorescence microscope.

Generation and Expression of Human pIgR Deletional Constructs—Human and mouse pIgR cDNA constructs were kindly provided by Charlotte S. Kaetzel, University of Kentucky. Primers and deletion constructs were designed according to the DNA sequence information in GenBankTM accession numbers X73079 (human pIgR) and NM-011082 (mouse pIgR). DNA fragments encoding pIgR were amplified by PCR using a human or mouse cDNA construct as a template according to the conditions described previously (51). All primers were synthesized by Sigma-Genosys (Woodland, TX). The sequences and locations of the primers used in this study are listed in Table I. In most primers, restriction sites were engineered at the 5′ ends to facilitate cloning of PCR products. PCR DNA fragments were digested with appropriate restriction enzymes and ligated into a mammalian expression vector pcDNA3.1(−), which drives protein expression with a cytomegalovirus (CMV) early promoter. The ligation mixtures were transfected in E. coli strain DH5α and selected with 100 μg/ml ampicillin by standard methods (47). The E. coli clones carrying correct recombinant plasmids were identified by PCR and restriction digestions. The identified constructs were verified by DNA sequencing. Protein and DNA sequence analyses were performed using GCG programs (Genetics Computer Group, Madison, WI).

To establish stably transfected cell lines in MDCK cells, plasmids containing correct inserts were transfected into cells using Superfection reagent according to the supplier’s manual (Quagen, Valencia, CA). The cells were then selected in the presence of 700 μg/ml G418 for 1–2 weeks. Individual clones were transferred to 24-well plates to prepare frozen stocks. The same clones were grown in chamber slides (Nalge Nunc International, Rochester, NY) to screen for expression of human pIgR by immunofluorescence microscopy using a rabbit anti-human SC serum. The cells were then treated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. The protein sizes and expression levels of human pIgR constructs were further verified by immunoblotting analysis of cell lysates as described previously (51). Mammalian cell lysates were prepared by using a Triton X-100 lysis buffer containing phenylmethylsulfonyl fluoride and a mixture of protease inhibitors (Roche Applied Science, Indianapolis, IN) (47). For certain human pIgR constructs (as specified under “Results”), recombinant proteins were expressed in COS-7 cells by transient transfection with 10–20 μg of plasmid DNA in a similar manner as the above stable transfection. The cells were lysed 24–48 h later, and pIgR expression was analyzed by immunoblotting.

Ligand Precipitation—CbpA-binding activities of pIgR, SC, and S-IgA were determined by ligand precipitation using CbpA-coated beads as described previously (30). Briefly, recombinant CbpA2 from serotype 4 TIGR strain (300 μg) or bovine serum albumin (BSA) was conjugated covalently to 107 carboxylated beads (Polysciences, Warrington, PA) according to the supplier’s instructions. CbpA2 was shown to contain a pIgR-binding site in our previous study (30). To perform ligand precipitation, CbpA and BSA-coated beads (~4 × 109) were diluted to 100 μl in a binding buffer (PBS, pH 7.4, 0.1% Triton X-100, 0.5 mM CaCl2) and mixed with 100 μl of the proteins (0.1–1 μg) or mammalian cell lysates for 1 h at room temperature. The beads were then washed three times in the binding buffer and resuspended in 15 μl of SDS-PAGE loading buffer. The bound proteins were separated in SDS-PAGE gels and detected by immunoblotting analysis. Mammalian cell lysates were prepared as described above. The beads were boiled to centrifugation at 14,000 rpm in a microcentrifuge for 10 min at 4 °C to remove insoluble cellular debris. The supernatants were preserved to perform ligand precipitation.

ELISA—The CbpA-binding activity of SC was quantified by using an
CbpA Binding Site on plgR

**FIG. 1.** CbpA binding to human S-IgA. CbpA2-coated beads were incubated with equal molar amounts of dimeric IgA1 (lacking SC) and S-IgA. Free IgA controls (lanes 1 and 2) and the bound IgA proteins (lanes 3 and 4) were separated by SDS-PAGE. The blot was reacted with a peroxidase-conjugated antibody against human IgA and visualized using an ECL method. Molecular mass markers are indicated in kilodaltons.

ELISA method as described previously (50). Recombinant CbpA2 (500 ng/well) was coated to the wells of 96-well plates (Nalge Nunc International) by incubating overnight at 4 °C. BSA was used as a negative control. After washing and blocking, biotin-labeled human free SC (100 ng/well) was added to the CbpA2-coated wells for 1 h at room temperature. Biotin labeling of SC was performed using an EZ-Link biotinylation kit from Pierce according to the supplier. The bound SC was detected using a 1:2000 (v/v) dilution of streptavidin-peroxidase conjugate (Pierce). Absorbance was read on a microtiter plate reader at a wavelength of 490 nm (Bio-Rad). The ELISA results are presented as the absorbance units after subtraction of the background readings that were determined by measuring the absorbances of the wells without biotin-labeled SC. To determine the effects of DTT,iodoacetamide, EDTA, and EGTA, these reagents were added at various stages of the absorbance reaction. The ELISA results are presented as the absorbance units after subtraction of the background readings that were determined by measuring the absorbances of the wells without biotin-labeled SC. To determine the effects of DTT, iodoacetamide, EDTA, and EGTA, these reagents were added at various stages of the absorbance reaction.

Structural Modeling of Human plgR—A model of domains D3/D4 of human plgR was built using the homology modeling technique. The Rutgers PDB data base was searched using the Protein BLAST search option (www.ncbi.nlm.nih.gov/BLAST) to select known structures that are related to the D3 or D4 domain. The variable domain of the light chain of the antibody against influenza virus hemagglutinin (PDB code 1CFT) (52) was selected for modeling the D3 domain of pIgR, the D4 domain was modeled using the variable domain of the light chain of the antibody against influenza virus hemagglutinin (PDB code 1QFU) (53). They share ~20% sequence identity with the D3 and D4 domains of plgR, respectively. The sequence alignments were performed using a GeneMine package (Molecular Application Group, Palo Alto, CA). The Model homolog module was individually used to model the D3 or D4 domain. After minimization, the two domains were combined and the SEGMOD module of GeneMine (54) was used to model the connection region between the D3 and D4 domains. Fig. 5E was generated by Molscript (55) and Raster3D (56).

**RESULTS**

CbpA and IgA Bind to Distinct Sites on Human plgR—Previous studies have shown that the Ig-like domain 1 of plgR is required for binding the J chain-containing polymeric IgA (41) and the C03 loop of IgA (38–40). Hammerschmidt et al. (21) reported that SpsA (a CbpA variant) binds to human S-IgA (21), indirectly suggesting that CbpA and IgA bind to plgR/SC via separate binding motifs. We determined the nature of interaction between CbpA and human S-IgA by ligand precipitation. A recombinant form of CbpA, designated CbpA2, was covalently coated to latex beads and used to interact with purified human S-IgA and polymeric IgA. After extensive washing, the binding of S-IgA or IgA was examined by immunoblotting using rabbit anti-human IgA. The results showed that CbpA binds to human S-IgA (Fig. 1, lane 3). In contrast, no CbpA binding was detected with polymeric human IgA (Fig. 1, lane 4). As a negative control, BSA-coated beads did not precipitate S-IgA or IgA (data not shown). The multiple bands as seen in Fig. 1 (lanes 1 and 2) indicate partial reduction of IgA proteins under these conditions. We conclude that CbpA and IgA bind to different regions of human plgR.

CbpA Does Not Bind to Mouse plgR, SC, and S-IgA—A previous study showed the lack of interaction between SpsA and mouse SC (43). We therefore sought to determine whether the whole mouse plgR was able to bind to CbpA. The entire coding sequence of mouse plgR cDNA was amplified using primers PR187 and PR177 (Table I) and cloned in XhoI and HindIII sites of expression vector pcDNA3.1(−). As a positive control, human plgR was amplified using primers PR171 and PR177 (Table I) and cloned in pcDNA3.1(−) in a similar manner. The constructs were transiently transfected in COS-7 cells, and the expression levels of mouse and human plgR were evaluated by immunoblotting using rabbit antisera against mouse or human plgR (Fig. 2A; data not shown). The cells expressing mouse and human plgR were lysed by a Triton X-100 lysis buffer (47) and used to assess CbpA-binding activity by ligand precipitation using CbpA2-coated beads. As experimental controls, recombinant mouse SC (Fig. 2A) as well as S-IgA and dimeric IgA (Fig. 2B) were detected by immunoblotting as described in the figure legends. Equal molar amounts of mouse and human free SC and S-IgA were also compared. As positive controls, human free SC and plgR exhibited strong CbpA-binding activities (Fig. 2C, left panel). In contrast, there were no detectable CbpA bindings by mouse plgR or free SC (Fig. 2C, middle panel) or S-IgA (Fig. 2C, right panel). These results confirmed that CbpA binds to human plgR/SC and showed no interaction with mouse plgR, SC, and S-IgA. For convenience, human plgR is referred to as plgR from here on.

Domain 3 Is Necessary for CbpA Binding—We focused our mapping analysis on the SC portion of plgR, because this region has been reported to contain the CbpA-binding activity (21, 30, 43). Human SC contains 603 amino acids consisting of 5 Ig-like domains and a 150-amino acid C terminus (37). SC lacks the last 143 amino acids at the carboxyl end of plgR, including the transmembrane and cytoplasmic domains. To identify the region(s) of SC important for CbpA binding, the plgR cDNA was engineered to delete individual domains by PCR amplification and DNA cloning.

As illustrated in Fig. 3A, four DNA constructs (SC-1 to SC-4) were generated that lack successively domain 1 (amino acids 1–130), domains 1 and 2 (amino acids 1–231), domains 1–3 (amino acids 1–339), and domains 1–4 (amino acids 1–463), respectively. To maintain the natural expression pathway of plgR, all constructs retained the N-terminal signal sequence, transmembrane domain, and cytoplasmic tail of plgR. The truncated fragments were cloned in the EcoRI/AluI site of pcDNA3.1 (Fig. 3B). The recombinant plasmids were transfected into MDCK cells for protein expression under the CMV promoter. Empty vector and the construct containing the intact plgR were used as negative and positive controls, respectively. Stable MDCK cell transfectants were selected with G418; the MDCK clones expressing the plgR truncates were identified by immunofluorescence staining (not shown) and immunoblotting (Fig. 3C). The migration of plgR truncates in SDS-PAGE gels were slower than predicted on the basis of the amino acid sequences due to the preserved glycosylation (44, 46). The cell lysates of the positive clones were used to determine CbpA binding with CbpA2-coated beads by ligand precipitation followed by immunoblotting. Latex beads coated with BSA were used as a negative control.

As controls, the cells expressing intact plgR showed strong CbpA binding, whereas no CbpA binding was detected with the cells transfected with vector only (Fig. 3D). The plgR truncates SC-1 and SC-2 lacking domains D1 and domains D1–2, respec-
In this study, we investigated whether both the C-terminal domains D4 and D5 were involved in CbpA binding. As illustrated in Fig. 4A, the C-terminal amino acids of the SC region were successively deleted in constructs SC-5 (residue 636), SC-6 (residues 469–637), SC-7 (residues 358–637), SC-8 (residues 240–637), and SC-9 (residues 137–637). The plgR cDNA fragments were amplified by PCR and cloned in pcDNA3.1(−). The sizes of the DNA constructs were verified by agarose gel electrophoresis (Fig. 4B). The nucleotide sequences were verified by DNA sequencing (data not shown). The DNA constructs were transfected in MDCK cells to create stable cell lines in the presence of G418. The positive clones expressing the chimeric proteins were identified by immunofluorescence (data not shown), and the sizes and levels of the plgR truncates were determined by immunoblotting analysis (Fig. 4C). CbpA-binding capacities of the plgR C-terminal truncates were determined by ligand precipitation using the cell lysates from the positive clones. The cells transfected with the empty expression vector did not show CbpA binding (Fig. 4D), whereas those expressing the intact plgR had strong CbpA binding. CbpA binding activities were also detected with the cells expressing all five domains (construct SC-5) or the first four domains (construct SC-6), indicating that domain D5 is not necessary for the ligand-binding activity. However, CbpA binding was undetectable for the plgR constructs lacking domain D4 (constructs SC-7, SC-8, and SC-9). Hence, the first three domains alone did not bind to CbpA under the experimental conditions, although domain 3, which was shown to be necessary for binding activity, is still present in the SC-7 truncates (Fig. 3D). These data showed that the D4 domain is as essential as domain D3 to ensure CbpA binding. The D3 and D4 Domains Are Sufficient for CbpA Binding—The results shown in Figs. 3 and 4 indicate that both domains D3 and D4 of plgR are necessary for CbpA binding. Domains 3 and 4 of plgR consist of 113 and 112 amino acids, respectively. To verify whether these two domains by themselves are sufficient to bind to CbpA in the absence of other domains, we cloned the plgR cDNA segment encoding domains D3 and D4 in pcDNA3.1(−) (Fig. 5A). Chimeric protein expression was assessed by immunoblotting (Fig. 5B). For an unknown reason, construct SC-12 (D4 alone) consistently yielded much lower levels of protein expression when it was transfected transiently into COS-7 cells (Fig. 5B) or stably into MDCK cells (data not shown). This low yield phenotype was highly reproducible when the D4 domain alone was constructed in expression vec-
tor pIRES1neo (Clontech, Palo Alto, CA) with or without the N-terminal signal sequence and C-terminal region (data not shown). It is highly likely that the D4 domain is unstable in the absence of other adjacent domains.

The cell lysates were used to determine CbpA binding by ligand precipitation. The volume of cell lysates from construct SC-12 was increased proportionally to compensate for low expression of domain D4. These experiments consistently detected CbpA binding for the construct SC-10 (Fig. 5C), demonstrating that domains D3 and D4 are sufficient for CbpA binding. In contrast, there was no detectable CbpA binding with SC-11 (D3 alone) or SC-12 (D4 alone). These results showed that the combined domains D3 and D4 of pIgR are necessary and sufficient for CbpA binding. Because mouse SC

![Diagram A](image1)

**Fig. 3.** N-terminal deletion of pIgR. A, map of the N-terminal domain deletions in pIgR. Constructs SC-1 to SC-4 encoding various pIgR domains were PCR-amplified using the primers indicated at the start and end of each construct. The intact coding sequence was amplified using primers PR171 and PR177. For membrane expression of the proteins, the 63-bp 5' terminus encoding the signal sequence of pIgR was constructed by annealing complementary oligonucleotides PR206 and PR207, and ligated to the 5' ends of all deletional constructs. The positive (+) and negative (-) ligand binding results in D are summarized in the right panel. B, cloning of pIgR cDNA constructs. The PCR products of pIgR cDNA were digested with EcoRI and AflIII and cloned in the EcoRI/AflIII-digested pcDNA3.1(-). The expression of recombinant proteins was driven by the CMV promoter. The sizes of the DNA inserts were verified by restriction digestion of the recombinant plasmids. The DNA fragments were separated in a 1% agarose gel and stained with ethidium bromide. The molecular sizes of DNA markers are marked in kilobases. C, expression of pIgR in MDCK cells. Stably transfected MDCK cells with the pIgR expression constructs were lysed to determine the expression of pIgR by immunoblotting. D, CbpA binding of pIgR truncates. The MDCK clones expressing pIgR truncates were lysed to determine ligand-binding activities by ligand precipitation as described under "Experimental Procedures." The cells transfected with empty pcDNA3.1 (Vector) or the entire pIgR construct (Intact) were included as negative and positive controls, respectively. These experiments were repeated with lysates of at least two positive clones for each construct.
and plgR did not bind to CbpA (Fig. 2C), we attempted to identify critical residues for CbpA binding by comparing the amino acid sequences of human and mouse D3/D4 regions (Fig. 5D). Two stretches of non-conserved sequences (residues 273–276 in D3 and residues 400–408 in D4) exposed on the surface in the domain model (Fig. 5E) might account for the differential interaction with CbpA. However, it has to be kept in mind that neighboring domains in plgR might contribute to the overall structure and thus to the proper spatial arrangement of the CbpA binding site.

CbpA-plgR Interaction Does Not Depend on Mg$^{2+}$/H$^{11001}$ or Ca$^{2+}$/H$^{11001}$—Previous studies have shown that protein interactions between bacterial pathogens and host receptors often require the involvement of Mg$^{2+}$ and Ca$^{2+}$ (57, 58). To determine whether Mg$^{2+}$ and Ca$^{2+}$ are required for binding between CbpA and plgR, the CbpA-binding activity of human free SC was assessed by ELISA in the presence or absence of Mg$^{2+}$ and Ca$^{2+}$. EDTA at a concentration of 10 mM has been shown to abolish binding between integrins and the invasin protein of Yersinia pseudotuberculosis (58). The same concentration of EDTA also blocks binding of E-cadherin to internalin of Listeria monocytogenes (57). The ELISA experiments showed that the presence of up to 10 mM EDTA did not significantly affect the CbpA-binding levels of human SC when compared with non-treatment controls (Fig. 6A). Similar experiments showed no significant effect for 10 mM EGTA (Fig. 6B). The above experiments suggest that Mg$^{2+}$/H$^{11001}$ and Ca$^{2+}$/H$^{11001}$ are not required for plgR-CbpA binding.

Disulfide Bonding of plgR Is Essential for CbpA Binding—In preliminary experiments, SC and plgR barely bound to CbpA when they were denaturated with SDS or reduced with β-mercaptoethanol (data not shown). This observation suggested that a specific conformation or structure in SC/plgR is required for CbpA binding. The SC portion of plgR contains 10 disulfide bonds formed by 20 cysteine residues (59). These cysteine residues are highly conserved in the plgR proteins from human, bovine, rabbit, mouse, and rat (60). There are four cysteines in
the D3 domain (at positions 257, 271, 279, and 325 of the mature protein), and four additional cysteines in the D4 region (at positions 371, 385, 395, and 441) (Fig. 5). These cysteine residues form intra-domain disulfide bonds in both D3 and D4 domains (59).

We sought to define whether disulfide bonding of pIgR/SC is necessary for CbpA binding by ELISA. We first determined if treating CbpA with reducing agents would alter CbpA-SC binding. CbpA2 coated on microtiter wells was treated with 0.1–10 mM DTT. After the removal of DTT, the wells were incubated with biotin-labeled human SC. Consistent with the lack of cysteine residues in the CbpA protein (GenBank™ accession number AE007507), treating CbpA with DTT did not significantly alter CbpA-SC binding (Fig. 7A). We then treated biotin-labeled human SC with various concentrations of DTT for 1 h at room temperature before adding to the CbpA-coated wells. The ELISA result showed a dose-dependent reduction in CbpA-SC binding (Fig. 7B). Because the CbpA binding motif was localized to the D3/D4 region of pIgR (Fig. 5C), the SC-10 construct representing this region was treated with DTT to...
values are presented as the means ± S.E. of the readings in the duplicate wells. B, EGTA treatment was performed as described in A.

determine the significance of disulfide bonding of the D3/D4 region in CbpA binding. In contrast to the untreated control (Fig. 7C, lane 3), treatment with 10 mM DTT resulted in an appreciable decrease in CbpA binding of the D3/D4 region (lane 4). Consistently, higher concentrations of DTT (50 and 100 mM) completely abolished CbpA binding of the SC-10 construct (Fig. 7C, lanes 5 and 6). These results strongly suggest that CbpA binding depends on disulfide bonding within the D3/D4 region of plgR.

A previous study indicated that an intra-molecular disulfide bond within human SC is dissociated to form two disulfides with dimeric IgA (61). Thus, it is possible that CbpA binding requires rearrangement of thiol groups on plgR. We reasoned that potential rearrangement of disulfide bonds would result in the transient formation of free thiol groups within plgR and blocking such thiol groups would abolish CbpA binding of plgR. We tested this possibility by treating biotin-labeled human SC with 0.5 mM iodoacetamide in a manner similar to the above DTT treatment. The ELISA result revealed that CbpA binding of human SC is not affected by iodoacetamide (data not shown), suggesting that intra-molecular rearrangement of disulfide bonds in SC is not required for CbpA binding.

DISCUSSION

S. pneumoniae is capable of binding to plgR, SC, and S-IgA via its surface-exposed protein CbpA or CbpA homologs in a human-specific manner (21, 30, 43). The binding interactions occur through the common SC module of these host proteins. A previous study by Hammerschmidt et al. (43) identified a hexapeptide linear motif on CbpA as the SC-binding site by mutagenesis and peptide mapping. In this study, we demonstrated that the D3/D4 region of plgR contains one or more CbpA binding motifs. The initial deletional analysis from both the C and N termini showed that this region of 225 amino acids is necessary for CbpA binding. Subsequent experiments demonstrated that neither D3 nor D4 alone was capable of binding to CbpA, whereas a fusion combining D3 and D4 was sufficient to ensure CbpA binding. The other three Ig-like domains (D1, D2, and D5) are not essential for this activity, because complete deletions of these domains did not affect our ability to detect CbpA binding. The lack of involvement in CbpA binding by D1 and D5 explains why SC is able to bind to two ligands (dimeric IgA and CbpA) simultaneously. Domains D1 and D5 but not D2–4 have been shown to be required for plgR or SC binding to dimeric IgA (38–40,42).

The existing evidence suggests that the CbpA-binding activity depends on one or more conformational binding motifs on plgR/SC. First, disulfide bonding in plgR/SC is required for CbpA binding. Disruption of disulfide bonds with the reducing agent DTT abolished the CbpA-SC interaction. We further demonstrated that disulfide bonding in the D3/D4 region is critical for CbpA binding. It is therefore likely that intra-molecular disulfide bonds in the D3/D4 region contributes to the CbpA binding conformation. Second, we observed that plgR/SC lost the CbpA-binding activity when subjected to SDS-PAGE under reducing conditions and blotting. This may be in part due to the disruption of disulfide bonding by the reducing agent, β-mercaptoethanol, in the protein sample-loading buffer. In contrast, reduced and denatured CbpA was still able to bind to biotin-labeled human SC (data not shown), in agreement with the reported linear SC-binding motif on CbpA (43).

The CbpA-binding activity of SC/plgR does not appear to require Mg2+ and Ca2+. Mg2+ and Ca2+ are required for ligand-receptor interactions in Y. pseudotuberculosis (58) and L. monocytogenes (62). Glycosylation of SC has been shown to be critical for binding to respiratory mucus (63) and Clostridium difficile toxin A (36). However, our previous study showed that carbohydrate residues on human SC are not essential for CbpA binding (30), suggesting that CbpA/plgRSC interaction is mediated by direct contact of amino acid residues. Other interactions between CbpA and plgR/SC may exist and influence the affinity and avidity of the protein binding. Our previous study identified two identical, yet independent, plgR/SC binding domains in CbpA (30). In fact, most natural CbpA variants examined thus far contain two identical plgR/SC binding sites (15, 64). Thus, a single CbpA protein may bind to two plgR/SC molecules. It is reasonable to postulate that two plgR/SC binding sites on CbpA may increase the ligand binding avidity when CbpA is expressed on the cell surface of S. pneumoniae.

The lack of CbpA binding by mouse plgR in this study has confirmed the previous findings that CbpA binding occurs only with human SC/S-IgA but not with the mouse, rat, rabbit, and guinea-pig homologs (30, 43). This species-specific binding may be conferred by amino acid sequence variations in the D3/D4 region of plgR. There is 67% amino acid sequence identity.
between the D3/D4 domains of human and mouse pIgR. According to our molecular modeling, two stretches of variable amino acids in the D3/D4 region are likely to be exposed on the surface of pIgR/SC and thus play a role in this species-specific binding. Although disulfide bonding of human SC was critical for CbpA binding, it should not be the cause of the CbpA-binding deficiency of mouse pIgR. All cysteine residues of pIgR are highly conserved among the five mammalian species reported thus far (60). This species-specific binding between human SC/pIgR and S. pneumoniae is reminiscent of another species-specific interaction between E-cadherin and L. monocytogenes (62). E-cadherin, a critical protein for epithelial junctions, serves as a receptor for Listerial invasion of human intestinal epithelial cells by binding to internalin, a surface-exposed protein of L. monocytogenes (57). A single proline residue in human E-cadherin accounts for specific interaction between internalin and human E-cadherin but not mouse and rat E-cadherins (62). Interestingly, transgenic mice expressing human E-cadherin have been shown to be more susceptible to listerial infection (65). Because S. pneumoniae is a natural pathogen for humans but not for rodents, CbpA interactions with pIgR, SC, and S-IgA may contribute to the host tropism of this pathogen.

Pneumococcal interactions with pIgR, SC, and S-IgA are very intriguing, because these host proteins are involved in mucosal immunity against microbial infections (37). Understanding molecular mechanisms of these binding interactions will facilitate future investigation into their potential contribution to bacterial pathogenesis and/or host immunity. Our previous study suggests that CbpA-pIgR interaction enhances pneumococcal colonization and dissemination by enhancing adhesion to and invasion of mucosal epithelial cells (30). However, it is now clear that pIgR knockout mice are not an appropriate model for testing this hypothesis in vivo due to the lack of in vitro binding interaction between mouse pIgR and CbpA. Domain swap of the D3/D4 region of human pIgR into the corresponding region of mouse pIgR would allow the establishment of appropriate mouse models for this purpose. These knock-in models can also be used to study the significance of CbpA interactions with SC and S-IgA in pneumococcal pathogenesis. CbpA-mediated binding to S-IgA may allow S. pneumoniae to gain access to IgA at the mucosal surfaces of the respiratory tract. Like other respiratory pathogens, S. pneumoniae express an IgA1 protease, which inactivates human IgA1 by proteolytic cleavage of the IgA1 hinge region (66). It is thus possible that CbpA binding to S-IgA enhances pneumococcal cleavage of IgA and thereby attenuates the adaptive immune response. Alternatively, pneumococcal binding to SC and S-IgA may block pneumococcal adhesion at the mucosal surfaces and thus serve as a function of host immunity against pneumococcal infection.

To understand the biological significance of pneumococcal interactions with pIgR, SC, and S-IgA, we must also consider CbpA interactions with other host factors, including complement C3 protein (23) and complement factor H (24, 25, 67). Binding to complement proteins has been described as a common strategy for many pathogenic bacteria to evade complement attack (68). Thus, it is logical to postulate that CbpA interactions with complement proteins may attenuate innate immune responses to pneumococcal infection. The contribution of CbpA binding to C3 and factor H to pneumococcal pathogenesis in the host remains to be determined. Taken together, previous studies have suggested CbpA as a master molecule to interact with both the innate and adaptive functions of the immune system. Identifying binding motifs on both CbpA and host factors will provide precise targets for further character-
ization of these interactions in the context of bacterial pathogenesis and host defense.

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REFERENCES