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Differential Role of CbpA and PspA in Modulation of In Vitro CXC Chemokine Responses of Respiratory Epithelial Cells to Infection with *Streptococcus pneumoniae*▽

Rikki M. A. Graham and James C. Paton*

School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia

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Respiratory epithelial cells play an active part in the host response to respiratory pathogens, such as *Streptococcus pneumoniae*, by releasing chemokines responsible for neutrophil recruitment. In order to investigate the role of specific pneumococcal virulence factors in eliciting CXC chemokine responses, type II pneumocytes (A549) and nasopharyngeal cells (Detroit-562) were infected with *S. pneumoniae* D39 or mutants lacking choline-binding protein A (CbpA), pneumococcal surface protein A (PspA), or specific domains thereof. In response to wild-type D39, both A549 and Detroit-562 cells showed a significant increase in CXC chemokine mRNA and interleukin-8 protein. This response was increased twofold when a *cbpA* deletion mutant (ΔCbpA) was used, suggesting that CbpA inhibits CXC chemokine induction. All three N-terminal domains of CbpA are required for this effect, as in-frame deletion of the respective region of *cbpA* had the same effect on the CXC chemokine response as deletion of *cbpA* altogether. Infection with a *pspA* deletion mutant (ΔPspA) led to a twofold decrease in the CXC chemokine response of A549 but not Detroit-562 cells, compared to infection with D39 at 2 h. Thus, PspA appears to have the ability to stimulate early CXC chemokine release from A549 cells. Deletion of the region of *pspA* encoding the first N-terminal α-helical domain reduced the ability of *S. pneumoniae* to elicit a chemokine response to the same degree as deletion of *pspA* altogether. Thus, the N termini of CbpA and PspA exert differential effects on CXC chemokine induction in epithelial cells infected with *S. pneumoniae*.

Streptococcus pneumoniae (the pneumococcus) is a leading cause of invasive infections, including pneumonia, bacteremia, and meningitis, as well as less serious but highly prevalent conditions, such as sinusitis and otitis media. The pneumococcal choline-binding surface proteins CbpA and PspA have an established role in pathogenesis as described previously (2, 5, 6, 18, 27, 31, 34). PspA inhibits complement activation (36) and has also been shown to bind human lactoferrin (15). The N-terminal α-helical region (amino acids [aa] 1 to 288) is thought to be responsible for the anticomplement functions of PspA (21); it also binds to lactoferrin via the C-terminal half of this α-helical domain (14, 15). The N-terminal domain has a structure that folds back on itself and so is divided into two regions. In *S. pneumoniae* D39, region 1 of the mature PspA α-helix comprises aa 1 to 146, and region 2 is from aa 147 to 288. The proline-rich region (aa 289 to 370) is believed to act as a flexible tether, connecting the α-helical region to the choline-binding domain of the molecule that attaches PspA to the surface of the pneumococcus via phosphorylcholine (ChoP) residues on cell wall teichoic acid and lipoteichoic acid (21).

CbpA promotes adherence to host cells by interacting with the polymeric immunoglobulin receptor (8), but it also has other functions, including binding to factor H (10, 11). CbpA is predicted to be an elongated molecule with an N-terminal region that is α-helical in structure and can be divided further into three distinct domains (19, 20, 24). The first 100 to 150 aa are hypervariable, although there is partial conservation among CbpA subtypes (7, 19). Downstream of this region, there are two direct repeats, which are highly conserved between strains and are reported to play a role in binding of CbpA to secretory immunoglobulin A (12, 13, 24). These are followed by a proline-rich region that in D39 shows a high degree of similarity to the proline-rich region of PspA (7). Antibodies directed at this region have been shown to be protective against infection (7).

The CXC chemokines are a small family of chemoattractant cytokines involved in leukocyte trafficking. CXC chemokine responses are important for the ability of the host to resist pneumococcal infection, and the dynamics of these responses have been studied using mouse intranasal challenge models. Bacteria move quickly to the lungs, where they can be detected almost immediately after infection (9). An early increase in the murine CXC chemokine macrophage inflammatory protein 2 (MIP-2) is seen in the lung tissue and bronchoalveolar lavage fluid, and this is accompanied by an influx of neutrophils to the site of infection (9). In mice that went on to recover, there was an early, sharp MIP-2 response that helped to overcome infection. This response was short lived, with levels of MIP-2 in the lungs of these mice returning to normal levels by 48 h postinfection. Mice that died had a delayed response that became prolonged due to an inability to control the numbers of pneumococci, with levels of MIP-2 remaining elevated in the lungs and blood until death (9). This prolonged inflammatory response and the accompanying presence of neutrophils presumably increase lung damage, contributing to mortality.

* Corresponding author. Mailing address: School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA 5005, Australia. Phone: 61-8-83035929. Fax: 61-8-83033262. E-mail: james.paton@adelaide.edu.au.

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TABLE 1. *S. pneumoniae* strains used in this study

Strain	Description	Source or reference
D39	Serotype 2 (NCTC 7466)	1
CbpA$^-$	D39 CbpA insertion-duplication mutant, Ery^r	4
ΔCbpA	*cbpA* deletion mutant of D39	This study
CbpAΔHyp	Hypervariable region of *cbpA* (nt 424–720) deletion mutant of D39	This study
CbpAΔSR1	Small repeat region 1 of *cbpA* (nt 721–1206) deletion mutant of D39	This study
CbpAΔSR2	Small repeat region 2 of *cbpA* (nt 1207–1548) deletion mutant of D39	This study
CbpAΔPro	Proline-rich region of *cbpA* (nt 1549–1761) deletion mutant of D39	This study
PspA$^-$	D39 PspA insertion-duplication mutant	27
pspA::*erm* mutant	*pspA erm* insertion mutant of D39	This study
ΔPspA	*pspA* deletion mutant of D39	This study
PspAΔh1	α-Helical region 1 of *pspA* (nt 210–657) deletion mutant of D39	This study
PspAΔh2	α-Helical region 2 of *pspA* (nt 658–1083) deletion mutant of D39	This study
PspAΔhelix	α-Helical region of *pspA* (nt 210–1083) deletion mutant of D39	This study
PspAΔpro	Proline-rich region of *pspA* (nt 1084–1329) deletion mutant of D39	This study

While leukocytes such as neutrophils and alveolar macrophages contribute greatly to the release of proinflammatory cytokines in the lungs in response to infection, respiratory epithelial cells are also able to contribute to this response. Infection with *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* led to an increase in the release of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-8 (IL-8) (another CXC chemokine), and IL-1β from type II pneumocytes (A549) (37, 38), and infection with *Haemophilus influenzae* and *S. pneumoniae*, both singly and in combination, led to an increase in IL-8 release from Detroit-562 and A549 cells (32). Thus, epithelial cells, which are the first to contact invading pathogens, are able to contribute directly to the inflammatory response by releasing chemokines that may recruit leukocytes to the site of infection, thereby facilitating clearance of the bacteria. There are some indications that *S. pneumoniae* may modulate the release of CXC chemokines from epithelial cells during infection, but the specific pneumococcal factors responsible for this activity remain to be determined. In this study, we have examined the role of CbpA and PspA in this process by measuring chemokine induction in A549 or Detroit-562 cells infected with derivatives of *S. pneumoniae* with various defined mutations in *cbpA* or *pspA*.

MATERIALS AND METHODS

Bacterial strains. The properties of *S. pneumoniae* strains used in this study are listed in Table 1. *S. pneumoniae* strains were routinely grown in Todd-Hewitt broth (Oxoid, Basingstoke, England) supplemented with 0.5% yeast extract at 37°C, without shaking, or on blood agar (BA). Erythromycin (Ery) was added to growth media where appropriate at 0.2 μg/ml.

DNA isolation and manipulation. The oligonucleotide primers used in this study are described in Table 2. *S. pneumoniae* chromosomal DNA was isolated, purified, and analyzed as described previously (28). DNA amplification was performed by high-fidelity PCR using an Expand Long Template PCR system (Roche Molecular Diagnostics, Germany). Overlap extension PCR was performed essentially as described previously (17). Amplification products were purified using an UltraClean PCR Clean-Up DNA purification kit (QIAGEN, Hilden, Germany).

Transformation of *S. pneumoniae*. Transformation of *S. pneumoniae* with PCR product was performed using competence-stimulating peptide 1 (16) as described previously (29).

Construction of in-frame *cbpA* and *pspA* deletion mutants of D39. To construct a *cbpA* deletion mutant of D39, overlap extension PCR was used to create a linear DNA fragment comprising the 5′ and 3′ flanking regions of *cbpA* but with the *cbpA* open reading frame (ORF) itself deleted. The *S. pneumoniae* R6 genome sequence (NC_003098) was used to design oligonucleotides that bound 1.8 kb upstream and 2.4 kb downstream of *cbpA* (RMAG7 and RMAG8) (Table 2). Oligonucleotides were also designed to bind at the beginning and end of the *cbpA* gene facing outwards, incorporating the start codon and the following two codons (RMAG6) and the stop codon with the preceding codon (RMAG5). Products of the PCRs using RMAG5 plus RMAG8 and RMAG6 plus RMAG7 contained overlapping, complementary sequences at one end as a result of extensions incorporated into the oligonucleotides, such that when both products were combined and used as a template in a PCR using the oligonucleotides RMAG7 and RMAG8, the overlapping sequences annealed, bringing the two products together. Thus, the flanking regions of *cbpA* were amplified together, with nucleotides (nt) 10 to 2100 of the *cbpA* ORF deleted. This product was transformed into the competent CbpA$^-$ mutant (D39 with an insertion-duplication mutation in *cbpA* [4]), and homologous recombination resulted in deletion of *cbpA* from the chromosome. Transformants were selected by loss of Ery resistance by replica plating on BA and Ery-BA plates, and deletion of *cbpA* was confirmed by PCR, sequence analysis (data not shown), and Western blotting (Fig. 1A). The confirmed *cbpA* deletion mutant was designated ΔCbpA.

For reasons that remain unclear, repeated attempts to construct a PspA deletion mutant by transformation of an insertion-duplication mutation in *pspA* (PspA$^-$) with an analogous DNA construct were unsuccessful. Deletion of the *pspA* gene in *S. pneumoniae* D39 was therefore achieved using a two-step process. The first involved interrupting *pspA* with an Ery resistance cassette. The Ery resistance gene (*erm*) from pVA891 (25) was amplified using the oligonucleotides RMAG12 and RMAG13 (Table 2). These oligonucleotides have tails that overlap with complementary tails on other oligonucleotides (RMAG14 and RMAG15) designed to bind within the *pspA* gene (designed using the D39 *pspA* sequence [accession no. M74122]) (Table 2). The regions of *pspA* from nucleotides 151 to 738 and 1075 to 1897 were amplified using the oligonucleotides IDPspAa and RMAG14 and RMAG15 and IDPspAb, respectively (Table 2). The tails on the oligonucleotides created extensions on the *erm* and *pspA* PCR products that overlapped such that when the products were combined, the three fragments could anneal. The entire fragment was then amplified by PCR with oligonucleotides IDPspAa and IDPspAb. The resulting product comprised the *pspA* regions spanning nt 151 to 738 and 1075 to 1897 flanking *erm*. This product was transformed into competent *S. pneumoniae* D39. Transformants were selected by plating on Ery-BA and confirmed by PCR, sequence analysis, and Western blotting with PspA-specific antiserum (data not shown). The resulting *erm* insertion mutant was designated *pspA*::*erm*. The second step used overlap extension PCR to create a PCR product containing the upstream and downstream regions of *pspA* but with the *pspA* ORF itself deleted. This was achieved using the oligonucleotides RMAG1 and RMAG3 (Table 2) to amplify a 1.9-kb fragment immediately upstream of *pspA*, incorporating the start codon and the first three codons of the *pspA* ORF. Primers RMAG2 and RMAG4 (Table 2) were also used to amplify a 1.3-kb fragment immediately downstream of *pspA*, incorporating the *pspA* stop codon. Products of both reactions contained overlapping, complementary sequences at one end, such that when both products were used as a template in a PCR using the oligonucleotides RMAG3 and RMAG4, the overlapping sequences annealed and the resulting 3.2-kb amplicon comprised the two flanking regions of *pspA*, with nt 13 to 1857 of the *pspA* ORF deleted. This product was transformed into *pspA*::*erm*, and transformants were screened for the loss of Ery resistance by replica plating onto BA and Ery-BA. Putative mutants were confirmed by PCR and sequence analysis (data not

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]	Location (accession no.)
IDPspAa	ACAAGTCTAGCCAGCGTCGCT	D39 *pspA* nt 151–171 (M74122)
IDPspAb	TATCTGATACTTTGAACCATTGGC	D39 *pspA* complementary nt 1874–1897
RMAG1	aatttaatgggcTTACTTATTCATCTAAATTTACCTCTTTT	R6 genome complementary nt 128339–128367 (NC_003098)
RMAG2	tagatgaataagTAAGCCGATTAAATTAAAGCATGTTAA	R6 genome nt 130213–130239
RMAG3	TTAGAACGGCTTAAAATCAGATATGA	R6 genome nt 126448–126473
RMAG4	CGCCACCTAGAACACTCTTCG	R6 genome complementary nt 131668–131687
RMAG5	ataaacatgtttGTAAACTAAACCTAATATAACTAGTTA	R6 genome complementary nt 1987525–1987552
RMAG6	ttaggtttagtttaCAAACATGTTTATTTCCTTCTATAT	R6 genome nt 1989641–1989667
RMAG7	GCTGCACCGATAGACAGACGC	R6 genome nt 1985081–1985101
RMAG8	TCCTTGACCATATCTGCTCACC	R6 genome complementary nt 1991426–1991447
RMAG12	tgaagaagtcgctGAAGGAGTGATTACATGAACAA	pVA891 *ery* nt 5103–5125
RMAG13	tggctcttcagcCTCATAGAATTATTTCCTCCCG	pVA891 *ery* nt 4363–4384
RMAG14	attcactccttcAGCGACTTCTTCAGCATCCAC	D39 *pspA* complementary nt 718–738
RMAG15	taattctatgagGCTGAAGAGCCATCGCAACCA	D39 *pspA* nt 1075–1095
PspA Helix1 1	tttagcttcttcTGCTCTTACAACAGTAGGCTG	D39 *pspA* complementary nt 199–209
PspA Helix1 2	gttgtaagagcaGAAGAAGCTAAAGCAAAATTAGAA	D39 *pspA* nt 658–678
PspA Helix2 1	tggtttttctggTAGTTTTTTAGTAAGTTCTGGTGC	D39 *pspA* complementary nt 634–657
PspA Helix2 2	actaaaaaactaCCAGAAAAACCAGCTCCAGCT	D39 *pspA* nt 1084–1104
PspA Pro 1	tttccagcctgtCTCATTAACTGCTTTCTTAAGGTC	D39 *pspA* complementary nt 1063–1083
PspA Pro 2	gcagttaatgagACAGGCTGGAAACAAGAAAACG	D39 *pspA* nt 1330–1350
PspA helix 1	tggtttttctggTGCTCTTACAACAGTAGGCTG	D39 *pspA* complementary nt 199–209
PspA helix 2	gttgtaagagcaCCAGAAAAACCAGCTCCAGCT	D39 *pspA* nt 1084–1104
CbpA hyp1	cttttctcctggCGCATGAACCACACTTCCCAT	D39 *cbpA* complementary nt 403–423 (AF068646)
CbpA hyp2	gtggttcatgcgCCAGGAGAAAAGGTAGCAGAA	D39 *cbpA* nt 721–741
CbpA sm rep1 1	ctttttttcctgaTTTCAATGTATCTTTTTTAAACTTCTC	D39 *cbpA* complementary nt 694–720
CbpA sm rep1 2	gatacattgaaaTCAGGAAAAAAGGTAGCAGAAGCT	D39 *cbpA* nt 1207–1230
CbpA sm rep2 1	ttgttcagctggTTTCAGGGATGAGCTTGGAAG	D39 *cbpA* complementary nt 1186–1206
CbpA sm rep2 2	tcatccctgaaaCCAGCTGAACAACCACAACCA	D39 *cbpA* nt 1549–1569
CbpA Pro 1	ttgtttccagccTTTTTCTTTAACTTTATCTTCTTCTG	D39 *cbpA* complementary nt 1523–1548
CbpA Pro 2	gttaaagaaaaaGGCTGGAAACAAGAAAACGGT	D39 *cbpA* nt 1762–1782
IL-8 Fwd	GAAGGAACCATTCTCACTGTGTGTA	IL-8 mRNA nt 75–99 (M28130)
IL-8 Rev	TTATGAATTCTCAGCCCTCTTCAAAAAC	IL-8 mRNA complementary nt 402–375
ENA-78 Fwd	GAACCCGCGACCGCTCGC	ENA-78 mRNA nt 62–79 (XM_003507)
ENA-78 Rev	AGAAAAGGGGCTTCTGGATCAA	ENA-78 mRNA complementary nt 393–372
GCP-2 Fwd	CTCCACCCAGCTCAGGAACC	GCP-2 mRNA nt 14–33 (XM_003502)
GCP-2 Rev	GAAAAGGGGCTTCCGGGTCCA	GCP-2 mRNA complementary nt 351–331
MGSA Fwd	AGCCACACTCAAGAATGGGCG	MSGA mRNA nt 304–324 (XM_003504)
MGSA Rev	TGGCATGTTGCAGGCTCCTC	MSGA mRNA complementary nt 758–777
MIP-2α Fwd	ATTTGTTAATATTTCTTCGTGATGACATATCA	MIP-2α mRNA nt 709–740 (X53799)
MIP-2α Rev	TCGAAACCTCTCTGCTCTAACAC	MIP-2α mRNA complementary nt 1010–1032
MIP-2β Fwd	AGAACATCCAAAGTGTGAATGTAAGG	MIP-2β mRNA nt 198–223 (X53800)
MIP-2β Rev	TCCTTTCCAGCTGTCCCTAGAA	MIP-2β mRNA complementary nt 458–479
GAPDH Fwd	TCCTTGGAGGCCATGTGGGCCAT	GAPDH mRNA nt 206–228 (XM_033258)
GAPDH Rev	TGATGACATCAAGAAGGTGGTGAAG	GAPDH mRNA complementary nt 445–421

[a] Nucleotides in lowercase are 5′ extensions enabling annealing of PCR products.

shown) and by Western blotting (Fig. 1B). The confirmed *pspA* deletion mutant was designated ΔPspA.

Construction of CbpA and PspA domain deletion mutants. A strategy analogous to that described for construction of ΔCbpA was employed to construct derivatives of *S. pneumoniae* D39 in which regions of *cbpA* encoding the hypervariable region (aa 1 to 100), the first of two small repeats (aa 101 to 260), the second of these repeats (aa 261 to 375), and the proline-rich region (aa 376 to 445) were deleted in frame. The oligonucleotides used are listed in Table 2, and a schematic representation of the CbpA derivatives expressed by these mutants is shown in Fig. 2A. In all mutants, the leader sequence and choline-binding domain were left unaltered to ensure correct export of the protein and binding to the pneumococcal surface. Deletion of the correct region of the gene was confirmed by PCR and sequence analysis (data not shown). For each mutant, expression of a truncated CbpA protein was confirmed by Western blot analysis using CbpA-specific polyclonal antiserum (Fig. 1C). Confirmed mutants producing CbpA lacking the hypervariable region, the small repeat region 1, the small repeat region 2, or the proline-rich region were designated CbpAΔHyp, CbpAΔSR1, CbpAΔSR2, or CbpAΔPro, respectively.

A similar series of in-frame domain deletion mutants was constructed in the *pspA* gene of *S. pneumoniae* D39. This involved overlap PCR using oligonucleotides listed in Table 2, followed by transformation of D39 *pspA*::*erm*. The two regions of the N-terminal α-helix (aa 1 to 146 and aa 147 to 288) were deleted individually and together, as was the proline-rich region (aa 289 to 370). The mutants were designated PspAΔh1, PspAΔh2, PspAΔhelix, and PspAΔpro, respectively (Fig. 2B). In all mutants, the leader sequence and choline-binding domain were left unaltered to allow for correct export of the protein and binding to the pneumococcal surface. For each mutant, deletion of the selected region of the *pspA* gene was confirmed by PCR and sequence analysis (data not shown). The production of truncated PspA protein of the expected size by the various mutants was confirmed by Western blot analysis with PspA-specific polyclonal mouse antiserum (Fig. 1D). Additional Western blot analyses indicated that none of the mutations constructed in *cbpA* or *pspA* had any effect on production of either the heterologous surface protein or the pneumococcal toxin pneumolysin (data not shown).

Cell culture. All tissue culture media and reagents were obtained from Gibco (Grand Island, N.Y.) unless otherwise indicated. A549 cells were routinely grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 50 IU of penicillin, and 50 μg/ml streptomycin. Detroit-562 cells were routinely grown at 37°C with 5% CO_2 in Eagle's modified essential medium supplemented with 10% heat-inactivated FCS, 1 mM

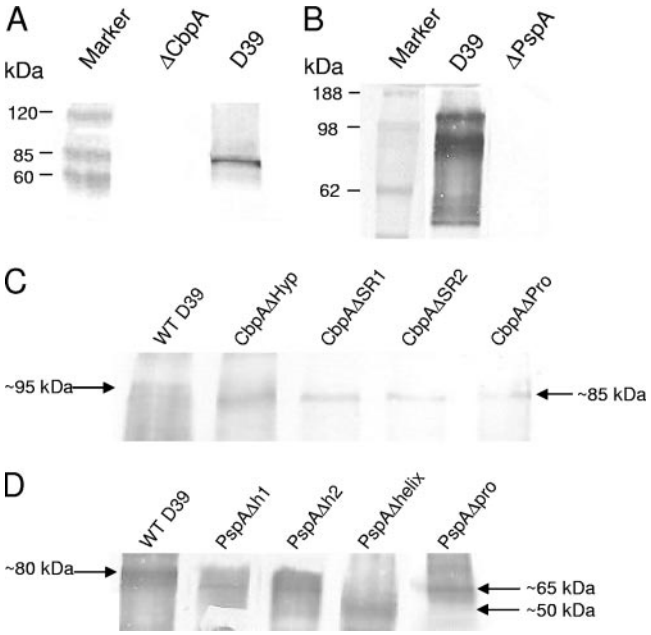

FIG. 1. Western blot analysis of CbpA and PspA mutants. Lysates of the indicated strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto nitrocellulose, and reacted with mouse polyclonal antiserum specific for CbpA (A and C) or PspA (B and D). A band of the appropriate size for CbpA (~95 kDa) was seen in the lysate of WT D39, while the sizes of bands seen in the lysates of the domain mutants were consistent with deletion of the specific domains (~85 kDa in each case). Lysates of WT D39 and *pspA* domain mutants were separated by SDS-PAGE, electroblotted onto nitrocellulose, and reacted with mouse polyclonal antiserum specific for PspA (as described in Materials and Methods). A band of the appropriate size for PspA (~80 kDa) was seen in the lysate of WT D39, while the sizes of bands seen in the lysates of the domain mutants were consistent with deletion of the specific domains (~65 kDa for the PspAΔh1, PspAΔh2, and PspAΔpro mutants and ~50 kDa for the PspAΔhelix mutant).

sodium phosphate, 1.8 g/liter sodium bicarbonate, 50 IU of penicillin, and 50 μg/ml streptomycin.

Infection of A549 and Detroit-562 cells with *S. pneumoniae*. For chemokine assays, A549 cells or Detroit-562 cells were seeded in six-well tissue culture trays and allowed to attach overnight. Cells were used at 90 to 100% confluence. Cells were washed twice with phosphate-buffered saline (PBS), 1 ml of the appropriate culture medium (without antibiotics or FCS) was added to each well, and cells were left to rest in air at 37°C (A549) or in 95% air-5% CO_2 at 37°C (Detroit-562) for at least 2 h. *S. pneumoniae* from overnight BA plates was inoculated into 10 ml Todd-Hewitt broth supplemented with 0.5% yeast extract and grown to an A_{600} of 0.15. *S. pneumoniae* cultures were then pelleted at 2,500 × *g* for 10 min, washed once, and then resuspended in 1.5 ml of the appropriate medium without FCS or antibiotics; 100 μl of this suspension (approximately 5×10^7 CFU) was used to infect cell culture monolayers. Two- and 4-h control cells received culture medium alone, with the 0-h control receiving nothing. Cell monolayers were then incubated in air at 37°C (A549) or in 95% air-5% CO_2 at 37°C (Detroit-562) for 2 or 4 h, at which time the supernatant was collected and stored at −20°C for analysis by IL-8 enzyme-linked immunosorbent assay (ELISA), and the monolayer was lysed in 1 ml TRIzol reagent for RNA extraction. Samples were also collected at 0 h to determine baseline chemokine expression in A549 or Detroit-562 cells before stimulation with *S. pneumoniae*.

IL-8 ELISA. Levels of IL-8 protein in the cell culture supernatant were measured using a commercial ELISA kit (R&D Systems Inc., Minneapolis, MI). Each well of a 96-well tray (MaxiSorp Nunc-Immuno plates; Nunc, Roskilde, Denmark) was coated with 200 ng of the capture antibody (monoclonal anti-human IL-8) diluted in PBS at room temperature overnight. Plates were washed three times with ELISA wash buffer (0.05% [vol/vol] Tween 20 in PBS, pH 7.4), using an ELx50 automatic strip washer (Bio-Tek Instruments Inc., Winooski, VA). Wells were then blocked by the addition of 300 μl blocking buffer (1% [wt/vol] bovine serum albumin [BSA], 5% [wt/vol] sucrose, and 0.05% [wt/vol] NaN_3 in PBS) and incubated at room temperature for 1 h before being washed three times. A total of 100 μl of culture supernatant or IL-8 standard (R&D Systems) diluted in ELISA diluent (0.1% [wt/vol] BSA, 0.05% [vol/vol] Tween 20 in Tris-buffered saline [20 mM Trizma base, 150 mM NaCl, pH 7.3]) was added to the wells. The plate was gently tapped for 1 min and incubated at room temperature for 2 h before being washed three times. A total of 100 μl of 20 ng/ml biotinylated goat anti-human IL-8 polyclonal detection antibody diluted in ELISA diluent was added to each well, and the plate was incubated for 2 h at room temperature before being washed a further three times. One hundred microliters of a 1/10,000 dilution of horseradish peroxidase-conjugated streptavidin diluted in Tris-buffered saline plus 0.1% (wt/vol) BSA was added to each well; the plate was incubated at room temperature for 20 min and then washed four times. Substrate solution (100 μl; one tablet each of the *O*-phenylenediamine and dihydrochloride tablet set [Sigma, St. Louis, MO] in 20 ml water) was added to each well and incubated in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4, and the A_{450} was determined using an ELISA plate reader (Dynatec MR5000).

RNA extraction and real-time RT-PCR. Whole cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) essentially as described previously (33). The comparative levels of chemokine mRNA produced by A549 and Detroit-562 cells after stimulation with various *S. pneumoniae* strains were determined using quantitative real-time reverse transcription (RT)-PCR as described previously (33), using oligonucleotide primer pairs specified in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control.

Statistical analysis. Statistical analysis was performed using Prism 3.03 software (GraphPad Software, San Diego, Calif.). Differences in chemokine responses between treatment groups were analyzed by one-way analysis of variance (ANOVA) with logarithmically transformed data; a P value of <0.05 was considered significant. When differences were significant, a Bonferroni test was performed.

RESULTS

CXC chemokine response of A549 and Detroit-562 cells to *S. pneumoniae* D39. In order to investigate the CXC chemokine responses, confluent monolayers of A549 and Detroit-562 cells were infected with approximately 5×10^7 CFU of *S. pneumoniae* D39 for 2 or 4 h before extraction of total cellular RNA and analysis of CXC chemokine-specific mRNA by real-time RT-PCR. The culture supernatant was also collected at 4 h and assayed for IL-8 by ELISA. At this dose, cells retained >95% viability, as judged by trypan blue exclusion (data not shown). At both time points, infected A549 cells exhibited a four- to fivefold increase in mRNA levels for IL-8 and MIP-2α, while MIP-2β mRNA increased approximately twofold compared to uninfected control cells. No obvious increases were seen in mRNA for ENA-78, GCP-2, or melanoma growth stimulatory activity (MGSA) at either 2 or 4 h postinfection (Fig. 3A). The concentration of IL-8 in *S. pneumoniae*-infected A549 culture supernatant (425 ± 75 pg/ml [mean ± standard error {SE}) was increased approximately sixfold relative to that of uninfected control cells (65 ± 15 pg/ml; $P < 0.05$).

Infection of Detroit-562 cells with *S. pneumoniae* D39 increased IL-8 mRNA approximately 8-fold at 2 h and approximately 60-fold at 4 h compared to uninfected control cells. MIP-2α mRNA was increased approximately 8-fold and 70-fold compared to that for uninfected control cells at 2 and 4 h, respectively. MIP-2β mRNA was increased approximately 4- and 29-fold, and MGSA mRNA levels were increased approximately 3- and 10-fold at 2 and 4 h, respectively. In contrast, levels of mRNA for ENA-78 and GCP-2 were not significantly affected by infection with *S. pneumoniae* (Fig. 3B). Levels of IL-8 protein secreted by Detroit-562 cells after exposure to *S.*

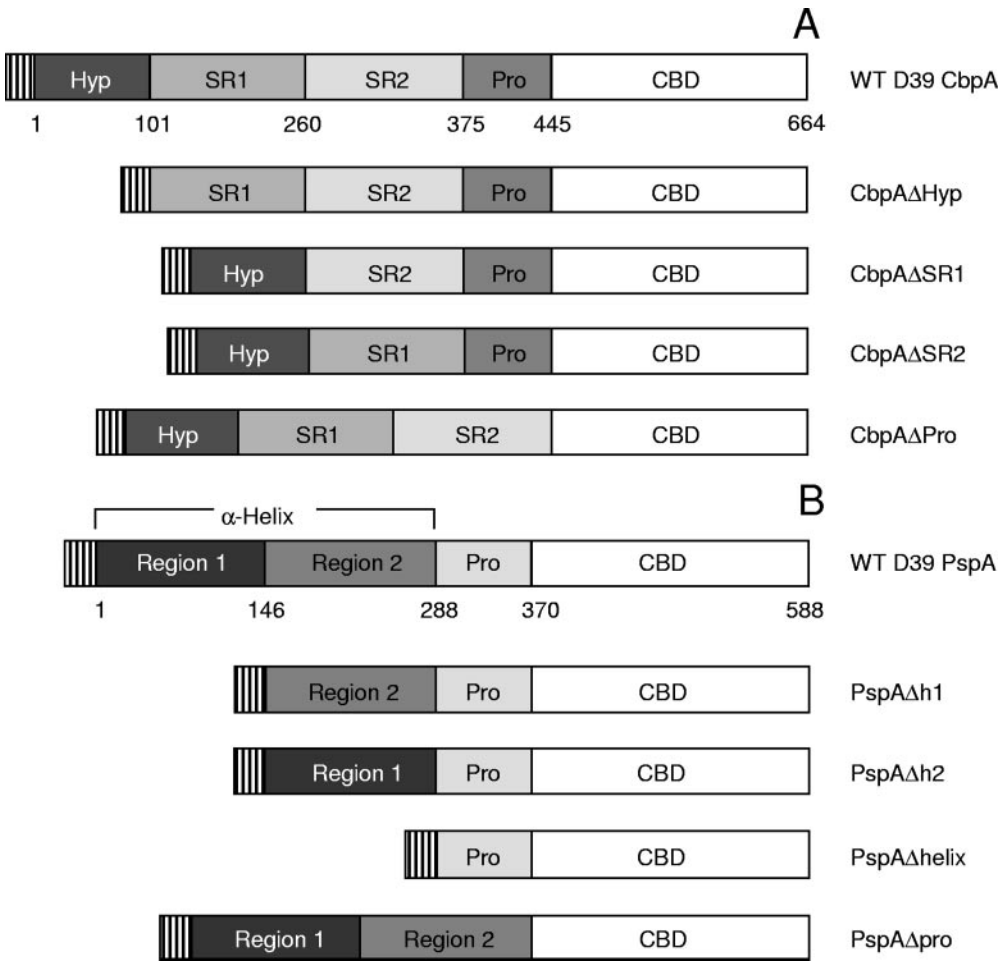

FIG. 2. Schematic representation of domain deletion mutants. (A) CbpA mutants. The leader sequence at the N terminus is hatched; other domains are designated as follows: Hyp, hypervariable region; SR1, small repeat region 1; SR2, small repeat region 2; Pro, proline-rich region; CBD, choline-binding domain. The numbers below the WT D39 CbpA map denote amino acid residues. (B) PspA mutants. The leader sequence at the N terminus is hatched; other domains are designated as follows: regions 1 and 2, respective portions of the α-helical domain; Pro, proline-rich region; CBD, choline-binding domain. The numbers denote amino acid residues in WT D39 PspA.

pneumoniae were increased approximately fivefold compared to those for uninfected control cells (1,474 ± 188 pg/ml and 279 ± 189 pg/ml, respectively; $P < 0.05$).

CXC chemokine responses of A549 and Detroit-562 cells to *cbpA* and *pspA* deletion mutants of *S. pneumoniae* D39. To investigate the role of the pneumococcal surface proteins PspA and CbpA in induction of chemokine responses, derivatives of D39 in which the genes encoding CbpA and PspA were deleted in frame (designated ΔCbpA and ΔPspA, respectively) were constructed, using a strategy analogous to that described by Berry et al. (4) for the pneumolysin gene (see Materials and Methods). A549 cells incubated with any of the strains showed a significant increase in both IL-8 and MIP-2α mRNA levels compared to uninfected control cells ($P < 0.001$). After 2 h, A549 cells showed an approximately twofold-greater increase in IL-8 mRNA when infected with the ΔCbpA mutant than with D39 ($P < 0.01$). At 4 h, levels of both IL-8 and MIP-2α mRNA were increased approximately twofold and fourfold, respectively, in ΔCbpA mutant-infected A549 cells, compared to those in cells infected with D39 ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 4A). However, infection with the ΔPspA mutant for 2 h led to IL-8 and MIP-2α mRNA levels in cells that were approximately twofold lower than those in cells infected with D39 ($P < 0.01$ for both), while at 4 h, mRNA levels were not significantly different (Fig. 4A).

Detroit-562 cells incubated with any of the strains also showed a significant increase in both IL-8 and MIP-2α mRNA levels compared to uninfected control cells ($P < 0.001$). In these cells, none of the mutants elicited a chemokine response significantly different from that elicited by D39 at 2 h. However, cells exposed to the ΔCbpA mutant for 4 h showed approximately two- and threefold increases in IL-8 and MIP-2α mRNA levels, respectively, compared to cells exposed to D39 ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 4B). The ΔPspA mutant did not elicit a chemokine mRNA response significantly different from that of Detroit-562 cells, compared to D39, at either 2 or 4 h (Fig. 4B).

At the protein level, both A549 and Detroit-562 cells showed a significant increase in IL-8 secretion when infected with any of the strains, compared to uninfected control cells ($P < 0.001$

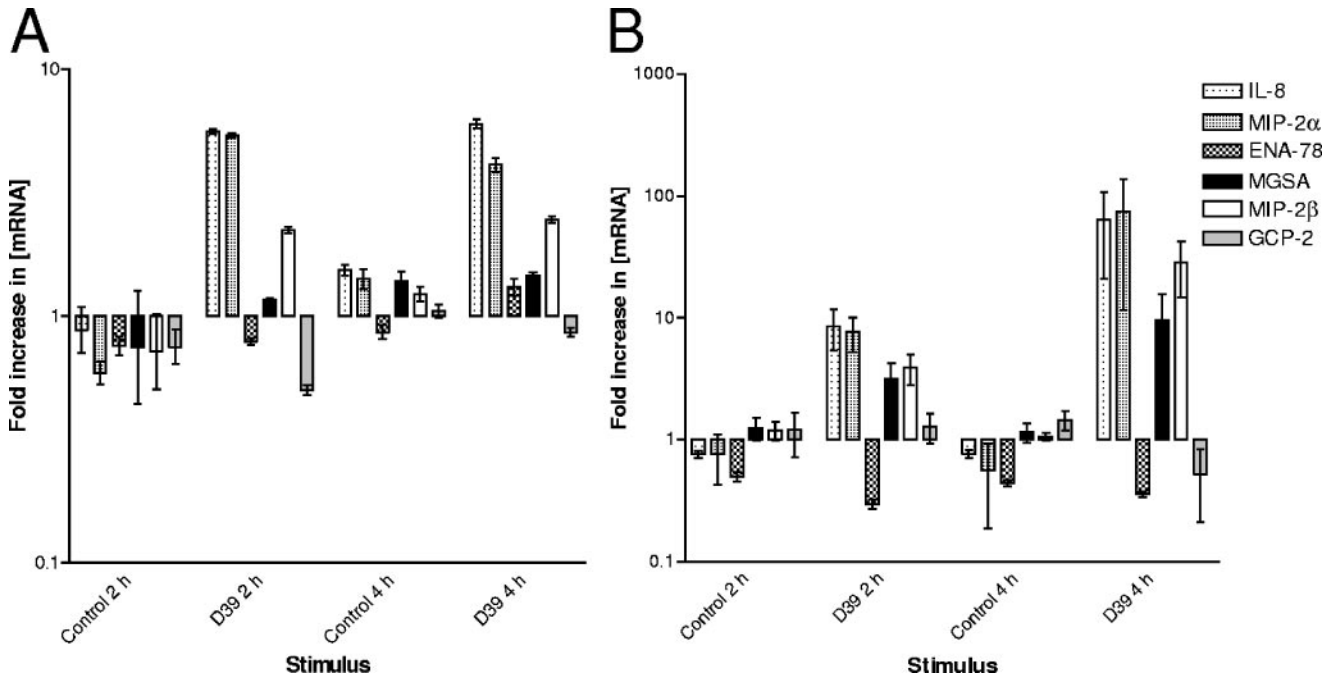

FIG. 3. CXC chemokine mRNA response of respiratory epithelial cells after infection with *S. pneumoniae* D39. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were infected with 5×10^7 CFU *S. pneumoniae* D39 for 2 or 4 h, at which time cellular RNA was extracted and analyzed by real-time RT-PCR, using oligonucleotides specific for IL-8, MIP-2α, ENA-78, MGSA, MIP-2β, and GCP-2. GAPDH mRNA was used as an internal control, and results are expressed as increases (*n*-fold) in mRNA at 2 or 4 h relative to an uninfected 0-h control.

for all) (Fig. 5). For A549 cells, IL-8 secretion was consistent with the mRNA levels measured above. Cells exposed to the ΔCbpA mutant secreted nearly twofold more IL-8 than those infected with D39 (910 ± 207 pg/ml compared with 551 ± 66 pg/ml; $P < 0.01$). IL-8 secretion from A549 cells exposed to the ΔPspA mutant (381 ± 46 pg/ml) was also significantly lower than for cells infected with D39 ($P < 0.05$) (Fig. 5A). Release of IL-8 by Detroit-562 cells also followed a trend similar to that seen for IL-8 mRNA, with the ΔCbpA mutant eliciting levels that were approximately twofold higher (1,828 ± 300 pg/ml) than those elicited by wild-type (WT) D39 (947 ± 50 pg/ml; $P < 0.01$). The ΔPspA mutant induced a response from these cells that was not significantly different from that for D39 (Fig. 5B).

CbpA domains responsible for modulation of the CXC chemokine response. To investigate whether specific domains of CbpA were involved in modulation of the chemokine response, regions of *cbpA* encoding the hypervariable region (aa 1 to 100), the first of two small repeats (aa 101 to 260), the second of these repeats (aa 261 to 375), and the proline-rich region (aa 376 to 445) were deleted in frame in *S. pneumoniae* D39 (Fig. 2 and see Materials and Methods). These mutants, designated CbpAΔHyp, CbpAΔSR1, CbpAΔSR2, and CbpAΔPro, respectively, were used to infect confluent monolayers of A549 or Detroit-562 cells along with D39 at a dose of 5×10^7 CFU. After 4 h, cells incubated with the CbpAΔHyp mutant showed an approximately twofold-greater increase in IL-8 mRNA and a fivefold-greater increase in MIP-2α mRNA than cells infected with D39 (IL-8, $P < 0.05$; MIP-2α, $P < 0.01$) (Fig. 6A). Increases in IL-8 mRNA levels were also seen in cells infected with the CbpAΔSR1 and CbpAΔSR2 mutants compared to those for cells incubated with D39, but this was statistically significant only for the latter ($P < 0.05$). However, MIP-2α mRNA responses of A549 cells infected with these mutants were approximately sixfold and threefold greater than for those infected with D39 ($P < 0.001$ and $P < 0.05$, respectively). No significant increase in either IL-8 or MIP-2α mRNA was seen in cells infected with the CbpAΔPro mutant compared to that of cells infected with D39. Incubation with the ΔCbpA mutant led to an increase of approximately twofold in IL-8 and fourfold in MIP-2α mRNA levels compared to incubation with D39, as seen previously ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 6A).

A similar trend was seen for Detroit-562 cells infected with the various mutants. At 4 h, levels of IL-8 mRNA were increased approximately twofold in cells exposed to the CbpAΔHyp, CbpAΔSR1, or CbpAΔSR2 mutant, compared to those in cells infected with D39 ($P < 0.01$, $P < 0.05$, or $P < 0.05$, respectively) (Fig. 6B). Comparable stimulation (approximately fourfold relative to D39) of IL-8 mRNA also occurred in cells exposed to the ΔCbpA mutant ($P < 0.05$). However, the CbpAΔPro mutant elicited levels of IL-8 mRNA similar to those elicited by D39 (Fig. 6B). Differences in levels of MIP-2α mRNA followed the same trends as those seen for IL-8 mRNA, but these differences did not reach statistical significance (Fig. 6B).

For both A549 and Detroit-562 cells, differences in IL-8 secretion from the different CbpA domain mutants were consistent with those observed at the mRNA level (Fig. 7A and B). Infection with the CbpAΔHyp, CbpAΔSR1, or CbpAΔSR2 mutant elicited a significantly increased IL-8 response in comparison to infection with D39, similar to the response elicited

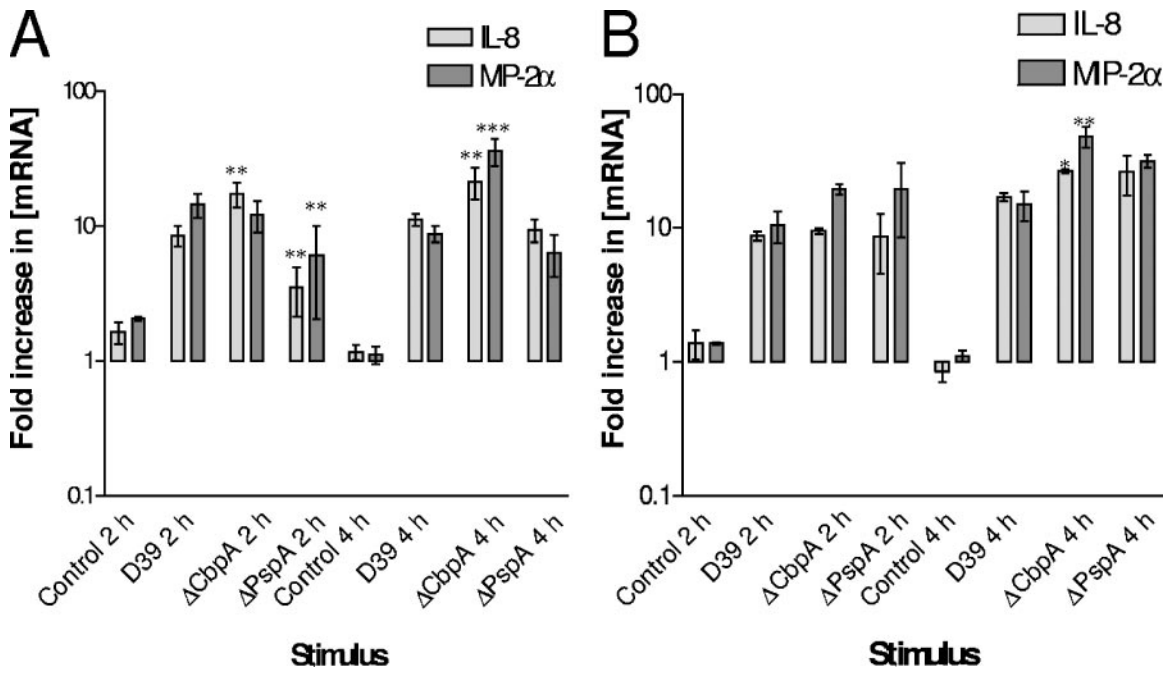

FIG. 4. CXC chemokine mRNA response of respiratory epithelial cells to *S. pneumoniae* D39, ΔCbpA mutant, or ΔPspA mutant. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with approximately 5×10^7 CFU *S. pneumoniae* D39, ΔCbpA mutant, or ΔPspA mutant for 2 or 4 h before extraction of cellular RNA and analysis of chemokine-specific mRNA by real-time RT-PCR. Results are expressed as increases (*n*-fold) of chemokine mRNA relative to a 0-h control. Experiments were performed in quadruplicate and analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (relative to D39 at the respective time point).

by the ΔCbpA mutant. The CbpAΔPro mutant elicited an IL-8 response similar to that elicited by D39 in both cell types (Fig. 7A and B).

PspA domains impacting the CXC chemokine response. A series of PspA domain deletion mutants was also constructed (Fig. 2 and see Materials and Methods). The two regions of the N-terminal α-helix (aa 1 to 146 and aa 147 to 288) were deleted individually and together, as was the proline-rich region (aa 289 to 370) (designated PspAΔh1, PspAΔh2, PspAΔhelix, and PspAΔpro, respectively). When A549 cells were infected with the various strains for 2 h, the PspAΔh1, PspAΔhelix, and ΔPspA mutants elicited IL-8 mRNA responses that were approximately two- to threefold lower than that elicited by D39 ($P < 0.05$ in each case). However, there was no significant difference between the responses elicited by the PspAΔh2 or PspAΔpro mutant and that elicited by D39 (Fig. 8A). Although MIP-2α mRNA responses showed a similar trend, none of the observed differences reached statistical significance (Fig. 8A). There was also no significant difference in the IL-8 mRNA responses generated by any of the mutants compared to that

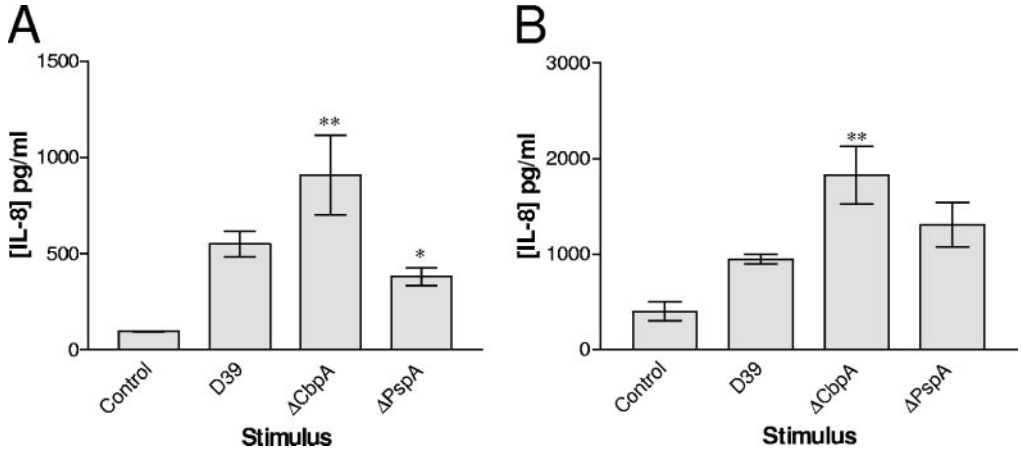

FIG. 5. IL-8 secretion from respiratory epithelial cells infected with WT D39, the ΔCbpA mutant, or the ΔPspA mutant. Cell culture supernatants from A549 (A) and Detroit-562 (B) cells infected with 5×10^7 CFU D39, ΔCbpA mutant, or ΔPspA mutant were assayed for IL-8 by ELISA. The data shown are the means ± SEs from three independent experiments. Results were analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. **, $P < 0.01$; *, $P < 0.05$ (relative to D39).

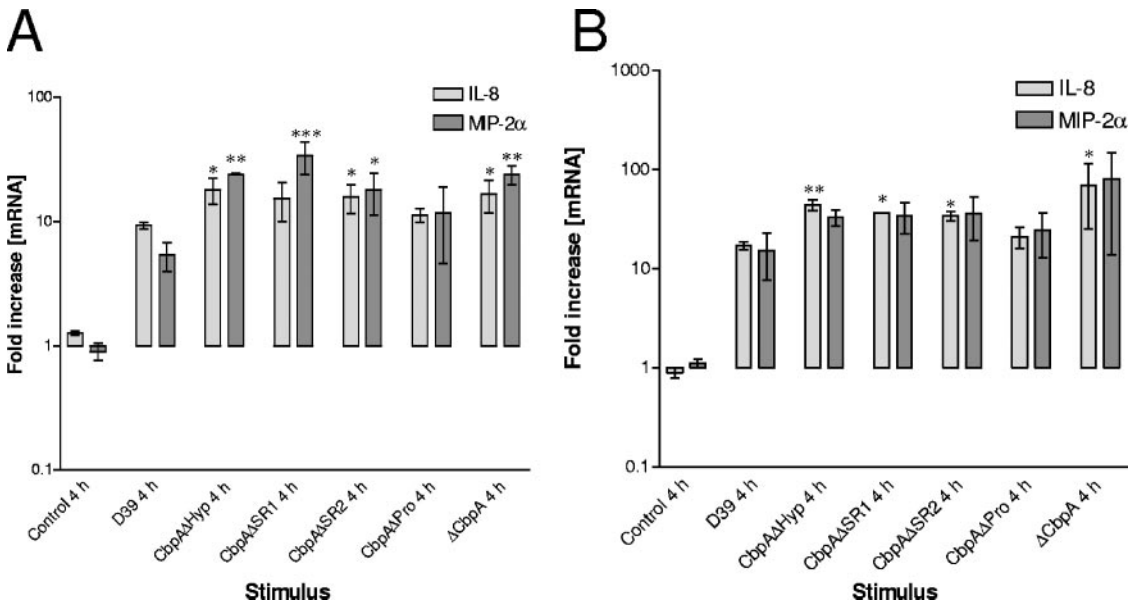

FIG. 6. CXC chemokine mRNA response of respiratory epithelial cells to CbpA domain mutants. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 5×10^7 CFU *S. pneumoniae* D39 or otherwise isogenic mutants, with in-frame deletions of regions encoding specific domains of CbpA, for 4 h before extraction of total cellular RNA and analysis of chemokine mRNA by real-time RT-PCR. Data are means ± SEs from three independent experiments. Data were analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ (relative to D39).

generated by D39 at 4 h (data not shown). Infection of Detroit-562 cells with the various PspA domain mutants did not result in significant differences in chemokine mRNA responses relative to infection with D39 at either 2 h (data not shown) or 4 h (Fig. 8B).

At the protein level, IL-8 secretion from A549 cells infected with either the PspAΔh1, PspAΔhelix, PspAΔpro, or ΔPspA mutant was significantly lower than that for cells infected with D39 ($P < 0.01$, $P < 0.01$, $P < 0.05$, or $P < 0.05$, respectively), while that for cells infected with the PspAΔh2 mutant was not significantly different (Fig. 9A). A similar reduction in IL-8 secretion from Detroit-562 cells was also seen in response to infection with the PspAΔh1 or PspAΔhelix mutant, compared to what was observed for infection with D39 ($P < 0.05$ in both cases) (Fig. 9B).

DISCUSSION

The initial results of this study demonstrate that infection of A549 cells with *S. pneumoniae* D39 leads to an increase in

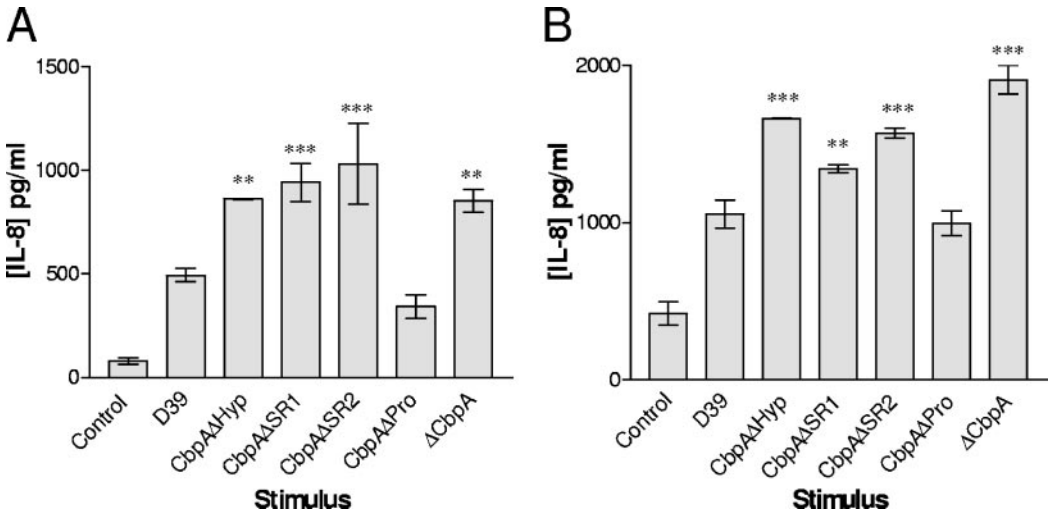

FIG. 7. IL-8 secretion from respiratory epithelial cells in response to CbpA domain mutants. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with *S. pneumoniae* D39 or CbpA domain mutants for 4 h before collection of the cell culture supernatant and analysis of IL-8 by ELISA. Data are means ± SEs from three independent experiments. Data were analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. ***, $P < 0.001$; **, $P < 0.01$ (relative to D39).

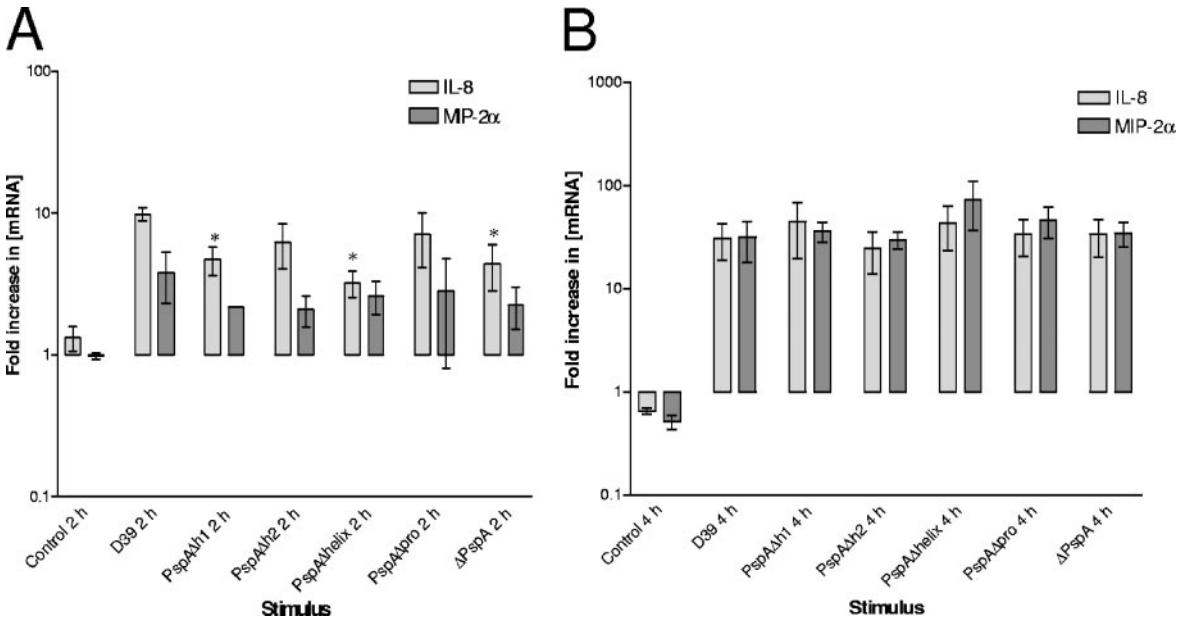

FIG. 8. CXC chemokine mRNA response of respiratory epithelial cells to PspA domain mutants. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 5×10^7 CFU *S. pneumoniae* D39 or otherwise isogenic mutants, with in-frame deletions of specific domains of PspA, for 4 h before extraction of cellular RNA and analysis of chemokine mRNA by real-time RT-PCR with specific oligonucleotides. Data are means ± SEs from three independent experiments. Results were analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. *, $P < 0.05$ (relative to D39).

mRNA for the CXC chemokines IL-8, MIP-2α, and MIP-2β and an increase in secretion of IL-8, compared to uninfected control cells. These results were consistent with previous in vivo studies that showed that levels of MIP-2 were increased in the lungs of mice infected with *S. pneumoniae* (3, 9, 22) and in vitro studies that showed an increase in IL-8 release by A549 cells in response to heat-killed *S. pneumoniae* (26, 30). Detroit-562 cells also responded to pneumococcal infection by increasing levels of IL-8, MIP-2α, MIP-2β, and MGSA mRNA and IL-8 protein.

We have also shown that CbpA and PspA have opposite modulatory effects on chemokine responses to *S. pneumoniae*. IL-8 and MIP-2α mRNA responses and IL-8 secretion levels were significantly higher when A549 or Detroit-562 cells were infected with the ΔCbpA deletion mutant than when cells were infected with wild-type D39. This suggests that CbpA suppresses the CXC chemokine response of respiratory epithelial cells, at least in vitro. Results obtained with a series of in-frame CbpA domain deletion mutants indicated that the three N-terminal domains (the hypervariable region and the two direct repeat regions SR1 and SR2) were all required for CbpA to have this effect. Mutants expressing CbpA in which any one of

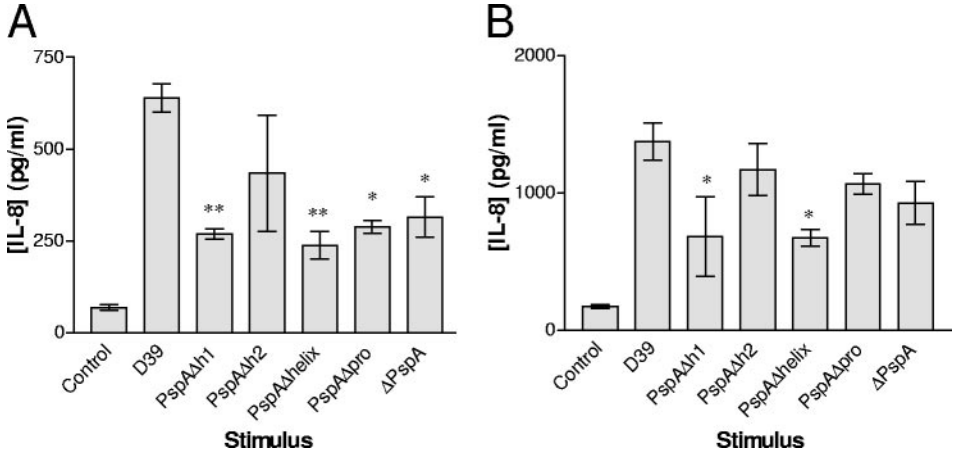

FIG. 9. IL-8 secretion from respiratory epithelial cells infected with PspA domain mutants. Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 5×10^7 CFU WT *S. pneumoniae* D39 or PspA domain mutants for 4 h before collection of the cell culture supernatant and analysis of IL-8 by ELISA. Data are means ± SEs from three independent experiments. Data were analyzed for statistical significance by one-way ANOVA with a post hoc Bonferroni test. **, $P < 0.01$; *, $P < 0.05$ (relative to D39).

these domains was deleted elicited a chemokine response from cells that was similar to that elicited by the ΔCbpA mutant. The exact mechanism by which these domains exert their suppressive effect remains unknown, but it may be dependent upon presentation of CbpA in a specific conformation that requires all three regions to be present. In contrast, the proline-rich region of CbpA appeared to have no suppressive effect on the chemokine response, with the CbpAΔPro mutant eliciting the same response from cells as D39. CbpA was able to suppress the CXC chemokine responses of both A549 and Detroit-562 cells, suggesting that the cognate receptor for this activity is common to both cell types. The ability of CbpA to suppress CXC chemokine production from nasopharyngeal epithelial cells may help the colonization of this site by preventing the recruitment of neutrophils that would otherwise clear the bacteria. In the lungs, an early sharp response has been shown to be vital in overcoming pneumococcal infection (9). Thus, CbpA-mediated attenuation of this early response may allow bacterial numbers in the lungs to reach a level that overwhelms the innate defense system, preventing clearance and leading to a sustained inflammatory response that aids the entry of pneumococci into the blood.

In contrast to the findings with the ΔCbpA mutant, infection of A549 cells with the ΔPspA mutant led to a twofold decrease in levels of IL-8 and MIP-2α mRNA at 2 h and IL-8 protein at 4 h, compared to infection with D39. Experiments with PspA domain deletion mutants showed that region 1 of the N-terminal α-helix of PspA was essential for the stimulatory activity of the molecule. Deletion of either region 1 or the entire α-helical region led to a twofold decrease in the IL-8 response of A549 cells; the PspAΔh1 and PspAΔhelix mutants elicited IL-8 responses that were similar to those elicited by the ΔPspA mutant. However, when region 1 was present and region 2 was deleted, the response elicited was not significantly different from that elicited by the wild-type strain. Deletion of the proline-rich region had no significant impact on IL-8 mRNA levels at 2 h, although there was a significant reduction in IL-8 secretion at 4 h. It is possible that deletion of this 82-aa tether region might impact surface exposure of the more critical α-helical region 1.

These results suggest that the α-helical region 1 of PspA has the ability to stimulate an early CXC chemokine response from type II pneumocytes, and this may contribute to the early inflammatory response towards *S. pneumoniae* in the lungs. Such responses would also be expected to result in recruitment of neutrophils and enhanced bacterial clearance. However, recent studies have shown that the level of transcription of *pspA* by *S. pneumoniae* in vivo is lower in the lungs than in the nasopharynx or blood (23), and this may limit the chemokine response at the former site. Interestingly, deletion of *pspA* had minimal impact on responses of Detroit-562 cells to *S. pneumoniae*, suggesting that PspA may not play a significant role in generating a CXC chemokine response from nasopharyngeal epithelial cells. PspA is reported to be important in protection of the pneumococcus from killing by apolactoferrin at mucosal sites, such as the nasopharynx (35), and transcription of *pspA* is increased in *S. pneumoniae* isolated from the nasopharynx of mice, relative to that from other in vivo sites (23). Our results suggest that increased expression of PspA in the nasopharynx might not lead to an increased inflammatory response, which could otherwise compromise survival of pneumococci in this niche.

A strength of the current study is that the role of CbpA and PspA in CXC chemokine induction in *S. pneumoniae*-infected epithelial cells has been examined using intact live bacteria, in which the proteins are presented in their native conformation and at physiological levels. Thus, the data are less susceptible to artifacts that might be induced by presenting the proteins at inappropriate doses or conformations. Nevertheless, we did examine the effect of treatment of A549 and Detroit-562 cells with purified CbpA and PspA (in the latter case, we used a 45-kDa N-terminal fragment to overcome solubility problems). At a dose of 10 μg/ml, both proteins elicited significant IL-8 responses in A549 and Detroit-562 cells, relative to responses elicited in untreated control cells. At least some of this stimulation may have been due to contaminating lipopolysaccharide, as the proteins were purified from recombinant *Escherichia coli*. However, for both cell types, even greater stimulation was seen when CbpA was heat inactivated. In contrast, heat treatment of PspA resulted in weaker responses (data not shown). These findings are compatible with those obtained using the *S. pneumoniae* mutants and support the conclusion that CbpA and PspA have opposite effects on CXC chemokine induction.

It is important to note that although PspA and CbpA have significant and differential effects on chemokine responses of respiratory epithelial cells in vitro, effects on other cell types present in the nasopharynx and the lungs could influence the overall host response to pneumococcal infection in a given niche. Moreover, neither CbpA nor PspA is solely responsible for induction or modulation of chemokine responses. None of the mutants tested reduced CXC chemokine induction from epithelial cells to control levels, and additional proinflammatory pneumococcal factors, such as lipoteichoic acid and pneumolysin, are likely to contribute. A better understanding of the process by which *S. pneumoniae* triggers an inflammatory response in the host will provide further insights into the early steps in pathogenesis of pneumococcal disease.

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