Calprotectin S100A9 Calcium-binding Loops I and II Are Essential for Keratinocyte Resistance to Bacterial Invasion*

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Epithelial cells expressing calprotectin, a heterodimer of S100A8 and S100A9 proteins, are more resistant to bacterial invasion. To determine structural motifs that affect resistance to bacterial invasion, mutations were constructed in S100A9 targeting the calcium-binding loops I and II (E36Q, E78Q, E36Q,E78Q) and the C terminus (S100A91–99 and S100A91–112), which contains putative antimicrobial zinc-binding and phosphorylation sites. The S100A8 and mutated S100A9 encoding plasmids were transfected into calprotectin-negative KB carcinoma cells. All transfected cells (except KB-sham) expressed 27E10-reactive heterodimers. In bacterial invasion assays with Listeria monocytogenes and Salmonella enterica serovar Typhimurium (Salmonella typhimurium), cell lines expressing S100A8 in complex with S100A9E36Q, S100A9E78Q, S100A91–99, or S100A91–112 mutants or the S100A91–114 (full-length) calprotectin resisted bacterial invasion better than KB-sham. When compared with KB-S100A8/A91–114, cells expressing truncated S100A91–99 or S100A91–112 with S100A8 also showed increased resistance to bacterial invasion. In contrast, glutamic acid residues 36 and 78 in calcium-binding loops I and II promote resistance in epithelial cells, because cells expressing S100A9E36Q,E78Q with S100A8 were unable to resist bacterial invasion. Mutations in S100A8 E36Q, E78Q were predicted to cause loss of the calcium-induced positive face in calprotectin, reducing interactions with microtubules and appearing to be crucial for keratinocyte resistance to bacterial invasion.

Mucosal keratinocytes continuously confront endogenous and exogenous invading microorganisms. Consequently the superficial keratinocytes of the oral mucosa contain a variety of indigenous bacteria (1). Yet the keratinocytes appear to resist large scale invasion and intracellular infection. Expressed in the cytoplasm of squamous mucosal keratinocytes, calprotectin (S100A8 and S100A9, MRP8 and MRP14, calgranulin A and B, L1, cystic fibrosis antigen, and 27E10 antigen) is a heterodimeric complex of polypeptides of 10.8 and 13.2 kDa, respectively (2–4). These two subunits are members of the S100 protein family, which are involved in cell cycle progression, cell differentiation, and cytoskeleton-membrane interaction (5–7). Calprotectin is the most abundant protein found in the cytoplasm of neutrophils (8, 9) and is also found in monocytes (10), macrophages (11), and human gingival keratinocytes (2). Elevated levels of calprotectin have been observed in body fluids such as plasma, saliva, gingival crevicular fluid, stools, and synovial fluid during infections and inflammatory conditions (12). Consequently, calprotectin is broadly used as a marker for inflammatory bowel diseases (13), reactive arthritis (14), and Sjogren syndrome (15).

Functioning as an antimicrobial protein (complex), calprotectin shows broad spectrum activities against microorganisms, including Capnocytophaga sputigena (16), Candida albicans (17), Escherichia coli, Staphylococcus aureus, Staphylococcus epidermis (18), and Borrelia burgdorferi (19). Calprotectin also inhibits bacterial invasion of epithelial cells by Listeria monocytogenes, Salmonella typhimurium, and Porphyrmonas gingivalis (20, 21). By promoting resistance to bacterial invasion, calprotectin-expressing cells, including squamous oral keratinocytes, are likely to contribute to mucosal innate immunity.

We have been studying the structural basis of calprotectin-mediated, cell-associated antimicrobial resistance. Unlike S100A8 and other members of the S100 family, S100A9 has an extended C-terminal region, which has an amino acid sequence (residues 89–108) that is identical to the N-terminal region of neutrophil immobilizing factor (22, 23) and homologous to domain 5 of high molecular weight kininogen (24). Domain 5 of high molecular weight kininogen has antimicrobial activity against E. coli, Pseudomonas aeruginosa, and Enterococcus faecalis (25). In addition, S100A9 C-terminal residues 103–105 form a polyhistidine motif (HHH), which may be involved in zinc binding (26, 27). Also suggested to be zinc-binding domains, the HXXXH motifs in S100A8 and S100A9 are commonly found in S100 proteins (4, 27, 28). Because zinc is required for bacterial growth, either the polyhistidine or HXXXH motifs have been suggested to bind and sequester zinc from microorganisms and inhibit bacterial growth (4, 27–29). In addition to zinc, calprotectin chelates other metal ions, including Mn2+ and Mn3+, which inhibits growth of S. aureus in tissue abscesses (30).

Independent of direct antimicrobial activity, epithelial resistance to invasion may also reflect the ability of bacteria to bind and internalize. Bacterial binding and internalization could be regulated by calprotectin as an interacting partner with the cytoskeleton, although distinguishing from antimicrobial activity may not always be clear. For example, S100A8/A9 translocates across the plasma membrane and is released from the cell...
in a tubulin-dependent manner (31). Release from the cell is controlled by the penultimate threonine (Thr-113) residue in the C terminus of S100A9, a substrate for protein kinase C (31). Although tubulin-dependent interactions may bring calprotectin in proximity to surface bacteria, these interactions could regulate cytoskeleton-dependent internalization (32).

In epithelial cells, calprotectin exists primarily as a heterodimeric complex of S100A8 and S100A9 and the individual subunits are not readily found (2). S100A9 integrity is critical to the formation of complexes with S100A8 (33) and the calcium-binding loops within the EF-hands contribute to intermolecular stability (4). The calcium-binding loops of S100 proteins also modulate intracellular calcium signaling, which affects cell differentiation, and cell cycle and cytoskeletal interactions (5). Integrity of the S100A9 calcium-binding loops may also be critical to resistance against bacterial invasion.

We considered that keratinocyte resistance to invasion reflected the ability of the cells to bind, internalize, and host viable invaders within the cell. In this study, we hypothesized that specific structural motifs of S100A9 in the calprotectin complex regulate epithelial cell resistance to bacterial invasion. To test this hypothesis, we designed five different S100A9 mutant constructs either in the calcium-binding or C-terminal domains using in vitro site-directed mutagenesis and deletion mutagenesis, respectively. Each mutated S100A9 was then expressed in KB cells with S100A8. As we reported previously (20), calprotectin (S100A8/A9) increased the resistance of epithelial cells to bacterial invasion. In the presence of S100A8, truncation of the C-terminal domain of S100A9 made the cells more resistant to invasion than with full-length S100A9. In contrast, mutations of S100A9 calcium-binding loops resulted in complete loss of resistance to bacterial invasion. Therefore, the central core polypeptide domain of S100A9 in the calprotectin complex plays a crucial role in epithelial resistance to bacterial invasion.

**EXPERIMENTAL PROCEDURES**

**Cells**—Wild-type calprotectin-negative KB cells (American Type Culture Collection, ATCC CCL-17) were maintained in modified Eagle’s media (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Mediatech) in 5% CO₂ at 37 °C. Transfected KB cells were maintained in modified Eagle’s media supplemented with 10% fetal bovine serum and 700 µg/ml G418 sulfate (Mediatech). To test the effect of calprotectin expression on viable bacteria, mutants and controls were maintained in medium without G418 sulfate for 4 days before the experiments were performed.

**Bacteria**—*L. monocytogenes* ATCC 10403S (provided by Dr. Daniel Portnoy, University of California, Berkeley) and *S. enterica* serovar Typhimurium (*S. typhimurium*) ATCC 14028 (provided by Dr. Roy Curtiss III, Washington University, St. Louis) were grown in brain heart infusion medium (Difco) and on tryptic soy agar (Difco) at 37 °C. *Listeria* and *Salmonella* were harvested from log phase or stationary phase, respectively (absorbance of 0.4 – 0.6 at 620 nm), and used to infect KB cells.

**Construction of Calprotectin and S100A9 Mutant Expressing KB Cells**—The structure of S100A8, S100A9, and mutant constructs in selected S100A9 functional domains are shown in Fig. 1, A and B. To construct S100A8 and S100A9 expression vectors, sequences were amplified using the following primers: S100A8, sense 5′-GGGGCAATTCAGCTGTGGGAC-3′ and antisense 5′-GCTTGCTATCAGCTTTTGTGGCTT-3′; S100A9, sense 5′-CGATGACCTTTGCAATGTCG-3′ and antisense 5′-GCCACTGTGGTCTTACGGGT-3′. To construct truncated S100A9 mutants (Fig. 1B), the sense primer was identical to S100A9 above, and the antisense primers were as follows: S100A9Δ1-112, 5′-TTAACCCTCCCGAGGGCTG-3′, and S100A9Δ99, 5′-TTAACCCTCTCGTCCAGCTTC-3′. S100A9 mutant sequences with point mutations in the calcium-binding loops, E63Q and E78Q (Fig. 1B), were constructed using the following oligonucleotides: S100A9E36Q, 5′-GCACCTGAACCAAGGGAATCTCGAAGCC-3′, and 5′-GCACCTGAACCAAGGGAATCTCGAAGCC-3′. S100A9ΔE78Q, 5′-GCAGCTGACTTTCCAGCTTCTCAGCTTC-3′, and 5′-GCAGCTGACTTTCCAGCTTCTCAGCTTC-3′, with the QuickChange® site-directed mutagenesis kit (Stratagene, Rockville, MD). S100A9ΔE36Q,E78Q was constructed using all the oligonucleotides from above. PCR products were cloned and amplified using pPCR-Script® (Stratagene, La Jolla, CA). All mutants were verified by sequencing. S100A8 and mutated S100A9 sequences were then cloned into pIRES (BD Biosciences) and pKN-1 (pIRES-EGFP; BD Biosciences with the BamHI site at 1887 bp attenuated) plasmids and co-transfected into KB cells using Superfect (Qiagen, Valencia, CA). Transfectants were selected by resistance to 700 µg/ml G418 sulfate and sorted for enhanced green fluorescent protein expression using a FACSorter (BD Biosciences). Cells co-transfected with insertless pIRES and pKN-1 served as a sham-control transfectant (KB-sham). Plasmids containing S100A8 and unmodified S100A9 were co-transfected into KB cells and served as a positive calprotectin-expressing control (KB-S100A8/A9Δ1-114). Stable transfectants were confirmed by reverse transcription-PCR using PCR primers listed above.

**Immunofluorescence**—Cells were grown on coverslips overnight, washed with PBS, and fixed with 4% paraformaldehyde for 10 min at room temperature. Monolayers were washed three times and permeabilized with 0.2% Triton X-100 for 2 min. After washing, monolayers were then incubated with murine monoclonal antibody against the calprotectin complex (mAb 27E10, diluted 1:50; Bachem, King of Prussia, PA) for 1 h at room temperature, followed by Alexa Fluor 568-conjugated goat anti-mouse IgG (diluted 1:500; Molecular Probes, Eugene, OR) for 1 h. Both antibodies were diluted in 3% (w/v) bovine serum albumin (Sigma) in PBS. The monolayers were washed and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). Slides were examined using a Nikon Eclipse epifluorescence microscope and photographed using a Spot digital camera (Diagnostic Instruments Inc, Sterling Heights, MI).

2 The abbreviations used are: PBS, phosphate-buffered saline; mAb, monoclonal antibody; CFU, colony-forming unit; PDB, Protein Data Bank; m.o.i., multiplicity of infection; ELISA, enzyme-linked immunosorbent assay.


**Calprotectin S100A9 and Bacterial Invasion**

**Sandwich ELISA**—To detect calprotectin complex, cells were resuspended in Hanks’ balanced salt solution (Invitrogen) and sonicated three times on ice at 50 watts for 15 s each (Sonifier Cell Disruptor W185, Heat Systems, Ultronics Inc., Plainview, MA). To obtain cell cytosol, sonicates were centrifuged at 10,000 × g for 20 min, and supernatants were collected, and total protein in each sample was determined by BCA protein assay kit (Pierce). Cell cytosol (50 μg) was analyzed for calprotectin using an ELISA. Briefly, 96-well plates were coated overnight at 4 °C with mAb 27E10 (diluted 1:100; Bachem), washed three times with PBS, pH 7.2, and 0.1% Tween 20, blocked for 1 h at 37 °C with blocking buffer (PBS, 0.1% Tween 20 and 0.5 mM CaCl₂), and washed three more times. Cell cytosol was added, incubated for 1 h at 37 °C, and washed three times. Biotinylated murine monoclonal antibody to S100A9 (S 36.48-biotin, diluted 1:200; Bachem) was then added and incubated for 1 h at 37 °C. Extravidin-horseradish peroxidase and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) were used and then centrifuged at 7500 g for 10 min. The supernatants were then washed and incubated with sterile distilled water for 15 min to release intracellular bacteria. Released bacteria were diluted, plated with a spiral plater (Spiral Biotech, Bethesda, MD), and incubated overnight at 37 °C, and the number of colony-forming units (CFUs) of internalized bacteria were enumerated on a New Brunswick C-110 colony counter (New Brunswick, NJ). The invasion assay was performed in triplicate and repeated at least three times.

**Immunofluorescence Analysis of Intracellular and Extracellular Listeria**—Cells (1.2 × 10⁵) were seeded on glass coverslips and grown overnight. As described previously (21), the monolayers were infected with *L. monocytogenes* for 2 h at an m.o.i. of 100:1, washed twice with Dulbecco’s PBS, and fixed with 4% paraformaldehyde. Extracellular Listeria were stained using rabbit anti-Listeria serum (diluted 1:3000; Biodesign, Kennebunk, ME) for 1 h, washed with PBS, and then incubated with Alexa Fluor 568-conjugated goat anti-rabbit IgG (diluted 1:500; Molecular Probes) for another hour. All antibodies were diluted in 3% bovine serum albumin in PBS. Cells were then permeabilized with 0.2% Triton X-100 for 2 min and then stained for both intracellular and extracellular Listeria. Permeabilized monolayers were washed, incubated with rabbit anti-Listeria serum for 1 h, washed three times, and then incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Molecular Probes) for 1 h. Nuclei were stained using 4′,6′-diamidino-2-phenylindole (diluted 1:3000; Molecular Probes). To verify antibody specificity, primary antibodies were replaced by rabbit serum. To determine nonspecific binding, secondary antibodies were added without primary antibody. Cells were observed using a Nikon Eclipse fluorescence microscope at ×400 magnification, and images from 20 random fields were captured with a Spot digital camera (Diagnostic Instruments Inc.). In each field, total Listeria (Alexa 488) and extracellular Listeria (Alexa 568) were counted. The number of intracellular Listeria was determined by subtracting the number of extracellular Listeria from the total count.

**Bacterial Binding Assay**—Binding of Listeria to KB cells was performed as described previously (21). Cells (1.2 × 10⁵) were...
seeded on glass coverslips and grown overnight. Monolayers were then incubated with *L. monocytogenes* at an m.o.i. of 100:1 for up to 60 min at 37 °C, washed, and fixed using 4% paraformaldehyde for 10 min. Adherent *Listeria* were labeled for 1 h with rabbit anti-*Listeria* serum (diluted 1:3000; Biodesign), washed, and incubated for 1 h with Alexa Fluor 568-conjugated goat anti-rabbit IgG. Separate coverslips were incubated with rabbit serum or secondary antibody as controls. At each time point, images from 10 random microscopic fields at ×200 magnification were captured with a Spot digital camera, and adherent bacteria were enumerated by visual counting.

**Structural Analysis of Calcium-free and Calcium-bound Calprotectin**—Because the structure of calcium-free calprotectin has not been determined, we generated the homology modeled structure using the program MODELLER (34). This program was chosen because of its ability to handle the alignment of a heterodimer target sequence (S100A8 and S100A9) with a homodimer structural template. Calcium-binding loops are in boxes. Putative zinc-binding domains are highlighted in gray. The phosphorylation site is boldface and underlined. Source, NCBI Entrez protein P05109 (A8) and P06702 (A9) (38). B, full-length S100A9 (S100A91–114) and S100A9 mutant constructs, including C-terminal domain deletions (S100A91–112 and S100A91–99) and amino acid substitutions in the calcium-binding loops (S100A9E36Q, S100A9E78Q, and S100A9E36Q,E78Q).

![Figure 1](image-url)
out as one file followed by removal of the information for calcium-free S100A8 and calcium-bound S100A9.

**Statistical Analyses**—Data are presented as the means ± S.E. Significant differences between control (KB-sham) and S100A9 mutants were determined using a two-sample Student’s *t* test. *p* < 0.05 was considered to be statistically significant.

**RESULTS**

**Formation of S100A8 and Mutant S100A9 Heterodimers**—As shown schematically in Fig. 1, KB cells were transfected to express calprotectin (S100A8/S100A9; Fig. 2B) and S100A8 in the presence of S100A9 C-terminal deletion mutants (Fig. 2, C and D) or point mutations in the calcium-binding loops (Fig. 2, E–G). Using complex-specific mAb 27E10, S100A8 in the presence of all mutant S100A9 variants appeared to form calprotectin complexes as suggested by immunofluorescence microscopy; KB-sham, the sham-transfected control cells, was negative (Fig. 2A).

Antigen(s) precipitated by mAb 27E10 were recovered from cytosol of clones expressing calprotectin or S100A8 co-expressed with mutant S100A9 and detected with biotinylated S100A9 mAb (S 36.48-biotin; Bachem) in sandwich ELISA (Fig. 3). Truncated variants of S100A9 co-expressed with S100A8 and calprotectin appeared to form similar amounts of calprotectin complex in KB cell cytosol and significantly more than the sham control (*, *p* < 0.05; **, *p* < 0.001; Fig. 3, A and B). In the same conditions, cells expressing S100A9 point mutations in calcium-binding loops did not appear to contain cytosolic heterodimers with S100A8 based on reaction with the anti-
S100A9 mAb (Fig. 3, C–E). To learn whether anti-S100A9 immunoreactivity was lost when point mutated S100A9 formed heterodimers with S100A8, immunoprecipitates were analyzed by SDS-PAGE and Western blotting (Fig. 4). On silver-stained gels, S100A8 and S100A9 (S100A91–114) proteins were visualized at 10.8 and 13.2 kDa, respectively (Fig. 4A). Recombinant proteins with similar molecular weights were detected in immunoprecipitates of S100A8 co-expressed with S100A91–112, S100A9E36Q, S100A9E78Q, and S100A9E36Q,E78Q; S100A8 with S100A91–99 was not well resolved. After immunoprecipitation with mAb 27E10, S100A8/A9 1–114, S100A8/A9 1–112, S100A8/A9 1–99, S100A8/A9 E36Q, S100A8/A9 E78Q, and S100A8/A9 E36Q,E78Q resolved on Western blots in reaction with anti-S100A8 (Fig. 4B). In contrast, anti-S100A9 antibodies failed to detect 27E10 immunoprecipitated S100A9E36Q or S100A9E36Q,E78Q (Fig. 4C) or E78Q (data not shown). To confirm that the anti-S100A9 antibody did not react with S100A9 calcium-binding loop mutants, cell lysates were analyzed directly by Western blotting. Lysates from all clones except the sham control reacted with anti-S100A8 (Fig. 4D). Anti-S100A9 reacted only with S100A9 and the C-terminal deletion mutants of S100A9 (S100A91–99 and S100A91–112), and failed to react when the calcium-binding loops were mutated (Fig. 4E). As expected, the calprotectin complex-specific mAb 27E10 did not react with either subunit of calprotectin in Western blots (data not shown). In general, clones producing the most heterodimers were chosen for further study. Clones with S100A9 calcium-binding loop point mutations were selected based on strong signals with 27E10 in immunofluorescence staining and anti-S100A8 after 27E10 immunoprecipitation.

**S100A9 C-terminal Deletion Increases Resistance to Bacterial Invasion**—To determine whether the C-terminal domain of S100A9 is crucial for cellular resistance to bacterial invasion, KB-S100A8/A91–112, KB-S100A8/A91–99, and control cells were incubated for 2 h with either *L. monocytogenes* ATCC 10403S (Fig. 5A) or *S. typhimurium* ATCC 14028 (Fig. 5B) at m.o.i. 100:1 or 1:1, respectively. When compared with the sham control, KB-S100A8/A91–114, KB-S100A8/A91–112, and KB-S100A8/A91–99 permitted significantly fewer viable intracellular *Listeria* (4.9 and 10.7% invasion, respectively) (*p* < 0.01). The numbers of internalized *Listeria* and *Salmonella* in calprotectin-negative cells (KB-sham) ranged from $1 \times 10^6$ to $3 \times 10^7$ CFU/well (1 ml) and from $2 \times 10^4$ to $4 \times 10^5$ CFU/well (1 ml), respectively. KB-S100A8/A91–99 cells showed the greatest resistance to invasion with viable intracellular *Listeria* and *Salmonella*, showing 7- and 5-fold fewer intracellular CFUs than in KB-sham. Remarkably, KB-S100A8/A91–112 and

**FIGURE 3. Calprotectin production in S100A9 mutants.** Calprotectin complex in KB-S100A8/A91–112 (A), KB-S100A8/A91–99 (B), KB-S100A8/A9E36Q (C), KB-S100A8/A9E78Q (D), and KB-S100A8/A9E36Q,E78Q (E) were estimated using a sandwich ELISA as described under “Experimental Procedures.” KB-sham and KB-S100A8/A91–114 cells were used as negative and positive controls. Values are means ± S.E. (*n* = 3, *, *p* < 0.05; **, *p* < 0.001). For each mutant, at least 10 clones were tested. Each experiment was performed in four replicates. The results are representative clones from each mutant.
KB-S100A8/A9,1–114 resisted invasion by *Listeria* more effectively than KB-S100A8/A9,1–114 (p = 0.002 and p = 0.02, respectively; Fig. 5A). KB-S100A8/A9,1–112 and KB-S100A8/A9,1–99 also appeared to resist *Salmonella* invasion more effectively than KB-S100A8/A9,1–114 (p = 0.03 and not significant, respectively; Fig. 5B).

To learn if the recovered intracellular CFUs reflected the total number of intracellular bacteria, *Listeria* was stained using a double immunofluorescence antibody approach. As expected, KB-sham cells contained more intracellular *Listeria* than the other clones. The number of intracellular bacteria in KB-sham cells was normalized to 100% invasion for each day’s experiment. When compared with KB-sham cells, the KB-S100A8/A9,1–114 cells contained 50% fewer intracellular *Listeria*; the C-terminal mutants, KB-S100A8/A9,1–112 and KB-S100A8/A9,1–99, each contained about 75% fewer intracellular *Listeria* (Fig. 5C).

**S100A9**, E63Q,E78Q Mutations Ablate Epithelial Resistance to Bacterial Invasion—To determine whether S100A9 calcium-binding loops I and II contribute to resistance to bacterial invasion conferred by calprotectin heterodimer, we quantified viable intracellular *Listeria* after invasion into KB-S100A8/A9,E63Q,E78Q, KB-S100A8/A9,E78Q, and KB-S100A8/A9,E63Q,E78Q cells. Consistent with the data above, KB-S100A8/A9,1–114 showed greater resistance to invasion by *Listeria* (50.3% invasion; p < 0.01; Fig. 6A) and *Salmonella* (59.1% invasion p < 0.001; Fig. 6B) than the KB-sham transfecant control. In identical conditions, calprotectin-negative KB-sham cells contained 1 × 10^6 and 3 × 10^7 CFU/well (1 ml) internalized *Listeria* and *Salmonella*, respectively. Not markedly different from KB-S100A8/A9,1–114, KB-S100A8/A9,E63Q and KB-S100A8/A9,E78Q hosted similar levels of invasion, showing 4-fold fewer viable intracellular *Listeria* (Fig. 6A) and 1.5–2-fold fewer *Salmonella* (Fig. 6B) than KB-sham. In contrast, KB-S100A8/A9,E63Q,E78Q cells, which have mutations in both S100A9 calcium-binding loops, fail to resist invasion by *Listeria* (99% invasion; Fig. 6A) and *Salmonella* (93% invasion; Fig. 6B) relative to KB-sham.

KB-S100A8/A9,1–114, KB-S100A8/A9,E63Q, and KB-S100A8/A9,E78Q showed similar percentages of total intracellular *Listeria* (ranges from 32.9 to 42%), when compared with KB-sham (Fig. 6C). Cells expressing calprotectin or single mutations in the calcium-binding loops of S100A9 co-expressed with S100A8 showed significant resistance to invasion (p < 0.01). Conversely, KB-S100A8/A9,E63Q,E78Q, which expressed S100A9 with mutations in both calcium-binding loops, showed a high level of intracellular *Listeria*, similar to KB-sham (Fig. 6C).

We next determined whether calprotectin-dependent resistance to invasion could be explained by differences in bacterial binding to the cells. Monolayers were incubated for 15–60 min with *L. monocytogenes* at an m.o.i. of 100. Cell-associated *Listeria* in nonpermeabilized cells were stained and counted as bound. For all tested KB cell lines, the numbers of bound bacteria increased with time. At all time points, significantly fewer *Listeria* bound to KB-S100A8/A9,1–114 and C-terminal mutants (KB-S100A8/A9,1–112 and KB-S100A8/A9,1–99; p < 0.05; Fig. 7A) or KB-S100A8/A9,E63Q (p < 0.05; Fig. 7B) than KB-sham. *Listeria* bound in similar numbers to KB-S100A8/A9,1–114 and C-terminal mutants (Fig. 7A). The number of *Listeria* bound to KB-sham and either KB-S100A8/A9,E78Q or KB-S100A8/A9,E63Q,E78Q was similar at all time points (Fig. 7B).

**Calcium-induced Conformational Changes in Calprotectin**—The predicted changes in calprotectin structure and charge upon calcium binding are shown in Fig. 8. This orientation was chosen to display the calcium-binding sites for both S100A8 and S100A9. The ribbon diagram of calcium-free calprotectin, generated by homology modeling, is shown in Fig. 8A. Calcium-free S100A8 (Fig. 8A, shown in yellow) and A9 (shown in green) contains four helices with the majority of the heterodimer interface formed by the interactions between the N- and C-terminal helices. The corresponding molecular surface for calcium-free calprotectin and the overall negative charge potential

**FIGURE 4. Analysis of S100A8 and mutated S100A9 in KB transfectants.** To analyze the heterodimeric complexes, the cell lysates (1 mg of protein) from KB-sham, KB-S100A8/A9,1–114, KB-S100A8/A9,1–112, KB-S100A8/A9,1–99, KB-S100A8/A9,E63Q, KB-S100A8/A9,E78Q, and KB-S100A8/A9,E63Q,E78Q were co-immunoprecipitated (IP) using mAb 27E10. The immunoprecipitated proteins were then separated by 15% SDS-PAGE and either silver-stained or electroblotted onto nitrocellulose paper (A) and detected with anti-S100A8 (B) and anti-S100A9 antibodies (C), as described under “Experimental Procedures.” Cell lysates were directly analyzed for S100A8 (D) and S100A9 (E) by Western blots (WB) as described under “Experimental Procedures.” Actin expression was used as protein loading control (lower panel in D).
(represented in red) is shown in Fig. 8B. By rotating this view 90° on the z axis, the S100A9 distal end of calprotectin and the overall negative charge are displayed in Fig. 8C. The ribbon diagram of calcium-bound calprotectin was generated based on PDB code 1XK4 and is shown in Fig. 8D. The heterodimer interface is conserved and consists primarily of the N- and C-terminal helices. Calcium binding, however, has induced the formation of an additional helix in the middle of the primary sequence, and these three helices are rotated relative to their position in calcium-free calprotectin. Additionally, the C-terminal helix expands by several turns upon calcium binding. Binding calcium changes the overall shape of calprotectin (Fig. 8E). The S100A8 calcium-bound structure combined with the E36Q,E78Q-mutated calcium-free S100A9 structure is shown as a ribbon diagram in Fig. 8G. The predicted effects of these mutations on the molecular surface are shown in Fig. 8H. When calcium is bound to calprotectin, the positively charged face of S100A8 appears unaffected by the E36Q,E78Q mutations in S100A9. However, the mutations in S100A9 result in the loss of the cleft in calprotectin as well as loss of a positively charged face on S100A9 (Fig. 8I).

**DISCUSSION**

We have previously reported that calprotectin can confer resistance to bacterial growth in the cytoplasm of intact cells (20), reduced bacterial binding to the cells, and significantly decreased invasion (21). How calprotectin protects and confers innate immunity to cells against invading microorganisms is not known. In vitro, calprotectin antagonizes the
growth of various microorganisms (16–18, 39). The mechanism of antimicrobial activity in vitro is not well understood, but calprotectin has been suggested to inhibit microbial growth by chelating Zn\(^{2+}\) using HXXH motifs commonly found in S100 proteins and HHH motif of residues 103–105 found in the C-terminal domain of S100A9 (27, 29). In this study, we characterized structural domains of S100A9 in association with S100A8 that are necessary to regulate epithelial cell resistance against invasion by L. monocytogenes and S. typhimurium.

Bacterial invasion depends on bacterial binding to the plasma membrane, subsequent cytoskeletal rearrangements to facilitate internalization, and intracellular survival. After invasion, keratinocytes are likely to harbor a mixture of live and dead intracellular bacteria. The antibiotic protection assay was used to estimate only viable intracellular bacteria that could be enumerated on agar, whereas immunofluorescence staining was used to directly visualize and count the total viable and nonviable bacteria within the keratinocytes. If a mutation abrogated antibacterial activity, a greater proportion of total intracellular Listeria (direct counts) was expected to be viable (estimated as CFUs). Indeed, with some exceptions, the CFUs recovered from the S100A9 mutants in this study mirrored the amount of visualized internal Listeria, suggesting that the ablated domains confer resistance to invasion through functions other than antibacterial activity (see Fig. 6). When the percentage reduction in viable Listeria exceeded the reduction in total bacteria, resistance to invasion may be attributable to intracellular antimicrobial activity and fewer Listeria entering the KB cells (see Fig. 5).

These data confirmed that deletion of the C-terminal domain of S100A9 when co-expressed with S100A8/A9E36Q and KB-sham. Other work from our group suggests that intracellular anti-Listeria activity might not be apparent until 5–7 h post-invasion (40). Consequently, the shorter term experiments (2 h post-invasion) we report here
Adherent bacteria were stained with specific antibodies and counted as

**Calprotectin S100A9 and Bacterial Invasion**

In S100A9 could disrupt calprotectin localization in the plasma membrane and dysregulate tubulin cytoskeletal events needed to bind and internalize bacteria.

The C terminus also includes a portion of a potential zinc-binding motif, \(^{106}\)XXH\(^{109}\) (Fig. 7A). When co-expressed with S100A8, S100A9\(_{1–112}\) and S100A9\(_{1–99}\) appear to reduce invasion similarly, and differences in presumptive intracellular anti-Listeria activity could not be detected. S100A9 has another potential component of a zinc-binding motif, \(^{97}\)HEXXH\(^{99}\), which could also contribute to antimicrobial activity (29). In vitro, these domains alone are not sufficient for antimicrobial activity because synthetic peptides containing HEXXH and HHX motifs did not inhibit *Candida* growth (29). Antibacterial activity attributable to the zinc-binding motifs could require the presence of the calprotectin complex. Yet S100 family members other than calprotectin show antimicrobial activity, including S100A7, S100A12, and S100A15 (45–47). These S100 protein family members do not possess an extended C-terminal domain with an HHX motif, nor do they appear to form heterodimers. Pretreatment of S100A7 with zinc did not impact antibacterial activity, and truncation of the C terminus of S100A7 to delete the zinc-binding HEXXH motif slightly reduced antibacterial activity in vitro (48). The central core domain of the S100A7 protein, which includes a functional EF-hand motif, showed full antibacterial activity, suggesting that the zinc-binding site in the C terminus of S100A7 may be necessary but not sufficient for antimicrobial activity. As recently reported, the antimicrobial activity of calprotectin may also depend on chelation of other metal ions such as Mn\(^{2+}\) (30), but other metal ion-binding motifs in the complex have not yet been determined. Hence, it is possible that other antimicrobial mechanisms may have been altered by the S100A9 mutations we report.

Complex formation by S100A8 and S100A9 could be necessary for antimicrobial activity and cellular resistance to invasion. Calprotectin complex formed with S100A9\(_{1–99}\) co-expressed with full-length S100A8 (Figs. 2–4 and Table 1). Similarly, murine S100A9\(_{1–101}\) (49), human S100A9\(_{1–101}\) (50) and human S100A9\(_{1–93}\) (24) each formed heterodimers with S100A8. Hence, heterodimerization into the calprotectin complex is independent of the extended C-terminal domain of S100A9.

Calprotectin complex formation with S100A8 appears to be stabilized by S100A9 through the C-terminal half of helix IV (adjacent to the C-terminal domain) or the hydrophobic amino acids in helix I (see Fig. 1) (9, 49, 51). Each S100 protein has two EF-hands. The canonical C-terminal EF-hand is formed by helices III and IV with an intervening calcium-binding loop; the N-terminal EF-hand contains a calcium-binding loop between helices I and II. Like other S100 proteins, calprotectin C-terminal EF-hands have an extended C-terminal domain with an HHH motif slightly reduced antibacterial activity

**FIGURE 7. Amino acid substitutions in the first and second calcium-binding loops of S100A9 decrease epithelial resistance to *Listeria* binding.** S100A9 C-terminal deletion mutants (KB-S100A8/A9\(_{1–112}\) and KB-S100A8/A9\(_{1–99}\)) (A) and S100A9 calcium-binding loop mutants (KB-S100A8/A9\(_{E36Q}\); KB-S100A8/A9\(_{E78Q}\); KB-S100A8/A9\(_{E36Q,E78Q}\)) (B) were incubated with *L. monocytogenes* ATCC 10403S for up to 1 h. Nonadherent bacteria were washed out, and the monolayers were fixed with 4% paraformaldehyde. KB-sham and KB-S100A8/A9\(_{1–114}\) were used as negative and positive controls, respectively. Adherent bacteria were stained with specific antibodies and counted as described under "Experimental Procedures." Values are means ± S.E. from three independent experiments. (*, *p < 0.05; **, *p < 0.01).

To study internalization may not be sufficient to observe the direct intracellular antibacterial effects of calprotectin. Nonetheless, when compared with KB-sham, calprotectin-expressing cells are more resistant to invasion by *Listeria* (4-fold greater) than *Salmonella* (2-fold greater). After invasion, *Salmonella* remains in vacuoles, whereas *Listeria* escapes from endosomes and resides in the cytoplasm (41) where cytoplasmic calprotectin would be encountered. Short term intracellular calprotectin-mediated anti-*Listeria* activity could contribute to the resistance to invasion seen in our assays.

We first tested KB cells that had been co-transfected with truncated C-terminal S100A9 constructs and full-length S100A8. The extended C terminus of S100A9 may actually enable invasion by *Listeria* and *Salmonella* differ, and the 2-h incubation time used to study internalization may not be sufficient to observe the direct intracellular antibacterial effects of calprotectin. Nonetheless, when compared with KB-sham, calprotectin-expressing cells are more resistant to invasion by *Listeria* (4-fold greater) than *Salmonella* (2-fold greater). After invasion, *Salmonella* remains in vacuoles, whereas *Listeria* escapes from endosomes and resides in the cytoplasm (41) where cytoplasmic calprotectin would be encountered. Short term intracellular calprotectin-mediated anti-*Listeria* activity could contribute to the resistance to invasion seen in our assays.

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antibody serves as a molecule probe for complex formation. S100A9 with mutated calcium-binding loops complexed with S100A8, because transfected KB cells reacted 27E10 as shown by immunofluorescence microscopy (Fig. 2) and immunoprecipitation of cell lysates (Fig. 4, A and B). Although calprotectin with mutated S100A9 calcium-binding Glu-36 and Glu-78 was

**TABLE 1**
Verification of calprotectin complex formation in S100A9 mutants

| Cell lines                  | ELISA<sup>a</sup> | IF<sup>b</sup> | WB<sup>c</sup> (anti-S100A8) | WB<sup>c</sup> (anti-S100A9) | IP<sup>d</sup> then WB (anti-S100A8) | IP<sup>d</sup> then silver staining |
|-----------------------------|--------------------|----------------|-----------------------------|-----------------------------|-----------------------------------|-----------------------------------|
| KB-S100A8/A9<sub>1–114</sub> | +                  | +              | +                           | +                           | +                                 | +                                 |
| KB-S100A8/A9<sub>1–112</sub> | +                  | +              | +                           | +                           | +                                 | +                                 |
| KB-S100A8/A9<sub>1–99</sub>  | +                  | +              | +                           | +                           | +                                 | +                                 |
| KB-S100A8/A9<sub>E36Q</sub>  | +                  | +              | +                           | +                           | +                                 | +                                 |
| KB-S100A8/A9<sub>E78Q</sub>  | +                  | +              | +                           | +                           | +                                 | +                                 |
| KB-S100A8/A9<sub>E36Q,E78Q</sub> | +              | +              | +                           | +                           | +                                 | +                                 |

<sup>a</sup> ELISA for calprotectin using mAb 27E10 as capture antibody is shown.

<sup>b</sup> IF indicates immunofluorescence staining for calprotectin using mAb 27E10.

<sup>c</sup> WB indicates Western blotting using anti-S100A8 and anti-S100A9, respectively.

<sup>d</sup> IP indicates immunoprecipitation using mAb 27E10 and then reacted with anti-S100A8 in Western blot or stained with methachromatic silver.

**FIGURE 8.** A representation of the changes in calprotectin structure and charge resulting from S100A9<sub>E36Q,E78Q</sub> mutations. The ribbon diagram of calcium-free and calcium-bound calprotectin, both wild-type and the mutant, is presented in A, D, and G, and the corresponding calculated charged molecular surface is shown in B, E, and H. The surface of S100A9 is shown in C, F, and I; the view was obtained by rotating the charged molecular surface of calprotectin 90° on the z axis, revealing the S100A9 underside. The location of C-terminal tail of S100A9 has not been resolved by crystallography and has been omitted from this model. A–C, model structure of calcium-free calprotectin. D–F, calcium-bound form of calprotectin, based on PDB code 1XK4 (4). G–I, S100A8 calcium-bound structure combined with the calcium-free S100A9 structure based upon the E36Q,E78Q mutations. Color key: S100A8, yellow; S100A9, green; calcium, pink; positively charged surface, blue; negatively charged surface, red; and hydrophobic surface, white.
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not detectable by a sandwich ELISA (Fig. 3, C–E), we show clearly that these mutations rendered S100A9 unable to react with anti-S100A9 antibody used for detection of calprotectin in this assay or S100A9 in Western blots (Fig. 4, C and E). Indeed, we screened several polyclonal and monoclonal anti-S100A9 antibodies, and all failed to detect S100A9 with mutated calcium-binding loops (Table 1). Nonetheless, anti-S100A8 antibody can detect S100A8 in the presence of S100A9 calcium-binding mutants (Fig. 3D and Table 1). Anti-S100A8 was not useful as a detection antibody for ELISA. In ELISA, this antibody was unable to detect calprotectin complex in KB-S100A8/A9E36Q,E78Q and KB-S100A8/A9E36Q (Fig. 7B), but low invasion (Fig. 6). The calprotectin S100A9 elimination of a cleft (Fig. 8E, arrow) formed in response to binding calcium. The adjustment of side chains to a new conformation alters the electrostatic potential of the molecular surface. In the case of calprotectin, the binding of calcium is responsible for creating a more positively charged surface.

Upon binding calcium, the molecular surface of S100A9 changes potential from negative to positive (Fig. 8, C and F), and the surface of calprotectin mirrors similar changes (B and E). The positively charged face of S100A8 in calprotectin that forms when calcium is bound may not be involved in bacterial invasion because this conformation is maintained upon E36Q,E78Q mutation (Fig. 8F).

The positive face of S100A9, however, may be critical to bacterial invasion (Fig. 8, C and F). The E36Q,E78Q mutations in S100A9 eliminate calcium binding and appears to lock S100A9 in a calcium-free conformation. S100A8 should bind calcium. The alteration of the molecular surface and the electrostatic potential provides several mechanistic explanations for how the calcium-binding domain could be involved in mediating resistance to bacterial invasion. The S100A9 E36Q,E78Q mutant loses interactions with any partner that requires a positively charged S100A9 functional surface. For example, negatively charged tubulin complexes with calprotectin and is involved in bacterial invasion (40). Because the E36Q,E78Q mutations in S100A9 result in loss of this interaction,3 we speculate that the positively charged surface of S100A9 contributes to interactions with tubulin cytoskeleton. Consistent with our structural predictions, calprotectin has been shown to contribute to cytoskeletal re-organization and microtubule polymerization in a calcium-dependent manner (4, 54, 57, 58).

Ca$^{2+}$-, Zn$^{2+}$-, and Cu$^{2+}$-binding motifs of S100 proteins generally regulate functional binding to effector molecules (5, 6). Calcium binding by calprotectin would be expected to affect concentrations of intracellular divalent cations, which can alter phosphorylation of specific molecules in downstream signaling cascades. Calprotectin may also interact with bacteria at the cell surface in a manner mimicking S100A12, which shows Ca$^{2+}$-dependent chaperone/anti-chaperone-like function (59). Calprotectin resistance to bacterial invasion therefore can involve complex downstream responses to calcium-dependent changes in structural motifs in S100A9 in oral keratinocytes.

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