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Molecules and Cells

Reduction-Sensitive and Cysteine Residue-Mediated *Streptococcus pneumoniae* HrcA Oligomerization *In Vitro*

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In both gram-positive and several gram-negative bacteria, the transcription of *dnaK* and *groE* operons is negatively regulated by HrcA; however, the mechanism modulating HrcA protein activity upon thermal stress remains elusive. Here, we demonstrate that HrcA is modulated via reduction and oligomerization *in vitro*. Native-PAGE analysis was used to reveal the oligomeric structure of HrcA. The oligomeric HrcA structure became monomeric following treatment with the reducing agent dithothreitol, and this process was reversed by treatment with hydrogen peroxide. Moreover, the mutant HrcA C118S exhibited reduced binding to CIRCE elements and became less oligomerized, suggesting that cysteine residue 118 is important for CIRCE element binding as well as oligomerization. Conversely, HrcA mutant C280S exhibited increased oligomerization. An HrcA double mutant (C118S, C280S) was monomeric and exhibited a level of oligomerization and CIRCE binding similar to wild type HrcA, suggesting that cysteine residues 118 and 280 may function as checks to one another during oligomer formation. Biochemical fractionation of *E. coli* cells overexpressing HrcA revealed the presence of HrcA in the membrane fraction. Together, these results suggest that the two HrcA cysteine residues at positions 118 and 280 function as reduction sensors in the membrane and mediate oligomerization upon stress.

INTRODUCTION

Heat shock proteins (HSPs), which are highly conserved in both prokaryotes and eukaryotes, are induced by various stresses such as elevated temperatures or exposure to ethanol or heavy metals (Morimoto et al., 1994; Neidhardt and VanBogelen, 1987). Among the various HSPs, Hsp60 and Hsp70 (GroEL and DnaK, respectively, in prokaryotes) play pivotal roles in the folding of both native and denatured proteins, and thus promote cell protection and survival (Gottesman et al., 1997).

A growing body of evidence indicates that the cellular response to heat stress is linked to changes in the physical state of membranes. Therefore, membranes can sense and transmit signals for HSP induction (Vigh et al., 1998; 2007). Indeed, the membrane fluidity of *Synechocystis* has been shown to be modulated *in vivo* by temperature acclimation, which is sensed and transduced into a cellular signal leading to the activation of heat shock genes (Horváth et al., 1998). Therefore, the membrane fluidizer benzyl alcohol (BA), a documented non-denaturant, generates and transmits stress signals to activate HSP genes in B16(F10) melanoma cells (Nagy et al., 2007). Moreover, HSP expression can be induced by even subtle abnormalities in the specific local nonbilayer microdomains of membranes (Horváth et al., 1998; Vigh et al., 2007).

The expression of the *groE* and *dnaK* operons in gram-negative organisms such as *Escherichia coli* is positively regulated by two alternative sigma factors, namely, σ^{32} and σ^{E} (Bukau, 1993). However, the expression of *groE* and *dnaK* operons in the gram-positive model organism *Bacillus subtilis* is negatively controlled by the HrcA repressor, which binds to the highly conserved CIRCE operator sequence (TTAGCACTC-N9-GAGTGCTAA) (Hecker et al., 1996; Narberhaus, 1999). The CIRCE/HrcA system has been identified in gram-positive bacteria as well as in some gram-negative bacteria (Ahmad et al., 1999; Narberhaus, 1999; Schulz and Schumann, 1996; Servant and Mazodier, 2001; Yuan and Wong, 1995). Most gram-positive bacteria, including *S. pneumoniae,* have the CIRCE sequence at the operator regions of the *groE* and *dnaK* operons, which are highly conserved and comprise *groES-groEL* and *hrcA- grpE- dnaK- dnaJ,* respectively (Kim et al., 2001; Schulz and Schumann, 1996; Yuan and Wong, 1995; www.tigr.org).

S. pneumoniae, which is carried in the nasopharynx of healthy individuals, is a major reservoir for pneumococcal infections. During invasion into the host, pneumococci are subject to environmental niche changes, i.e., from the nasopharynx (30 to 34°C) into the blood stream (37°C), which can trigger dramatic changes in morphology as well as gene expression. In fact, the elevated temperatures they encounter within a mammalian host induce HSPs (Choi et al., 1999). Further, during an *S. pneumoniae* infection, the levels of HrcA, pneumolysin, and PspA increase in the blood and resemble planktonic growth, whereas the expression of oxidative stress and competence genes, neuralminidases, and metalloproteinases increases in tissues such as

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the brain and lungs, where pneumococci exist in a biofilm-like state (Oggioni et al., 2006). Moreover, in *Mycoplasma pulmonis*, *hrcA* mutations increase resistance to antimicrobial peptides (Fehri et al., 2005), and HrcA in *Listeria monocytogenes* is regulated by σ^B (Hu et al., 2007). Thus, HrcA appears to play significant roles in both virulence and antibiotic resistance.

HrcA depends on GroEL for binding to CIRCE (Martirani et al., 2001; Mogk et al., 1997; Reischl et al., 2002), and GroEL is titrated by nonnative proteins in the cell (Mogk et al., 1997). Further, the helix-turn-helix motif of HrcA has been identified and shown to be involved in DNA binding (Hitomi et al., 2003; Liu et al., 2005; Wiegert and Schumann, 2003). HrcA functions as a thermosensor, detecting temperature changes in cells (Hitomi et al, 2003; Watanabe et al., 2001). Moreover, the dimeric form of HrcA displays sequence-specific DNA binding activity (Martirani et al., 2001). Previously, we demonstrated that Ca^{2+} represses the expression of the *dnaK* and *groE* operons *in vivo*, as well as the thermo-resistance of *S. pneumoniae*. Binding of HrcA to CIRCE is enhanced by Ca^{2+} (Kwon et al., 2005); however, the low solubility and easy aggregation of HrcA have prevented detailed clarification of this protein, and only a few *in vitro* biochemical studies concerning HrcA proteins have been reported (Minder et al., 2000; Roncarati et al., 2007; Susin et al., 2004; Wilson and Tan, 2002). Therefore, the specific mechanism that modulates the activity of HrcA remains unknown.

In this study, we show that HrcA is monomeric under reducing conditions, and that Ca^{2+} stimulates its oligomerization. Furthermore, the cysteine residue at position 118 appears to be important for binding to CIRCE elements as well as for oligomerization in *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and materials

S. pneumoniae CP1200 (a derivative of Rx1; Morrison et al., 1983), and the CP1200-derived *hrcA* mutant (Kim et al., 2001) were grown at 30°C as described previously (Morrison et al., 1983). The *E. coli* BL21 (DE3) (Novagen) strain was grown in Luria-Bertani (LB) broth or LB agar. For overexpression of HrcA, the *hrcA* ORF in pET32a (pKHY114) was amplified in *E. coli* BL21 (DE3), and HrcA was induced by the addition of 0.004 mM IPTG as previously described (Kwon et al., 2005). Cells were lysed in lysis buffer (50 mM Tris·Cl [pH 8.0], 0.1% Triton X-100, 1 mM PMSF), and the supernatant was employed for electromobility shift assays (EMSA). The supernatant was also used for HrcA purification using a modified method detailed in the manufacturer's instructions as previously described (Kwon et al., 2005).

Oligomerization analysis of HrcA by SDS-PAGE

The oligomerization of HrcA in the presence of Ca^{2+} was examined with 7.5% SDS-PAGE. HrcA (4 μg) was preincubated with the indicated concentrations of Ca^{2+} in 20 mM Tris-HCl (pH 7.5) for 30 min at 37°C. The reaction was further incubated with the coupling agent 0.1 mM 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide (EDAC) for 30 min at 37°C. The reaction was terminated by mixing the sample with 5× sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue), followed by boiling for 5 min. The samples were fractionated by 7.5% SDS-PAGE according to the Laemmli method (1970).

Construction of the HrcA deletion mutants

Deletion mutants of *hrcA* were produced by amplifying the *hrcA* region using primers specific to defined regions of the chromosomal DNA, which served as the template. The primer sequences are as follows; PTH1 (5′-GAG TCT ATT AAC TCT AGC AGT GCA-3′ and 5′-ATC GGG ATC CTT AAT GTA CTT CGT AAT GAT TAC TGC-3′), PTH2 (5′-GAG TCT ATT AAC TCT AGC AGT GCA-3′ and 5′- CGG GAT CCT TAT GTA AAG TAA CGC TGG ATA ATC TG-3′), PTH3 (5′-GTG GAT CCA AAG CCT TTG ATC AGG AAT TCT TC-3′ and 5′-CGC GTC GAC TTA ATG TAC TTC GTA ATG ATT ACT G-3′), PTH4 (5′-GTG GAT CCC GTT TGA CAG CCT TTG ATA TCG TT-3′ and 5′-CGC GTC GAC TTA ATG TAC TTC GTA ATG ATT ACT G-3′), PTH5 (5′-CGT GGA TCC ATG GTT ACA GAG CGT CAG CAG GAT-3′ and 5′-ACG CGT CGA CGG ATT TGA TTG ATT AGC TGT TGG TA-3′), PTH6 (5′-CGT GGA TCC ATG GTT ACA GAG CGT CAG CAG GAT-3′ and 5′-ACG CGT CGA CGA TTG GCA AAA TTC AAG AGA TTG AC-3′), PTH7 (5′-CGT GGA TCC ATG GTT ACA GAG CGT CAG CAG GAT-3′ and 5′-ACG CGT CGA CGG ATG TGT TCA AAG AGA TCG ATG AC-3′), PTH8 (5′-GTG GAT CCA AAG CCT TTG ATC AGG AAT TCT TC-3′ and 5′-ACG CGT CGA CGG ATG TGT TCA AAG AGA TCG ATG AC- 3′), PTH9 (5′-GTG GAT CCA AAG CCT TTG ATC AGG AAT TCT TC-3′ and 5′-ACG CGT CGA CGA TTG GCA AAA TTC AAG AGA TTG AC-3′). After amplification of the specific *hrcA* regions, PCR products were digested with either *Bam*HI and *Eco*RV (PTH1, 2, 3, 4) or *Bam*HI and *Sal*I (PTH5, 6, 7, 8, 9) and ligated into the vector pET-32(a), which had been previously digested with the same set of restriction enzymes (Promega) to produce the *hrcA* deletion mutants.

Localization of HrcA

To determine the localization of HrcA in *E. coli* cells, HrcA without a histidine-tag was overexpressed and proteins were processed into periplasmic, membrane, and cytoplasmic fractions as described previously (Van Dijl et al., 1991). HrcA without a His-tag was amplified using the following primers: 5′-GGC CCA TAT GGT TAC AGA GCG TCA GCA GGA TAT TT-3′ and 5′-ATC GGG ATC CTT AAT GTA CTT CGT AAT GAT TAC TGC-3′ and ligated into a pET-30(a) vector which had been digested with *Nde*I and *Bam*HI to produce the recombinant plasmid pKHY105. To fractionate proteins, 100 ml of culture was harvested and the cell pellet was resuspended in 10 ml of spheroplast buffer (30 mM Tris-Cl, 20% sucrose [w/v], 10 mM EDTA, 0.5 mg/ml of lysozyme) and incubated at 37°C for 30 min. After centrifugation at 13,000 × *g* for 20 min, the supernatant was taken as the periplasmic fraction and the pellet was resuspended in 1 ml of spheroplast buffer and 19 ml of hypotonic buffer (0.1 M Tris-Cl pH 7.6, 1 mM PMSF, and 1 mM EDTA) followed by incubation on ice for 10 min. The reaction mixture was further centrifuged at 5,000 rpm for 10 min and the supernatant was harvested as the cytosolic fraction. The remaining pellet was then resuspended in 500 μl of solubilizing buffer (0.05 M Tris-Cl pH 8.0, 5 mM NaCl, 0.4% Triton X-100, 0.05% Sarkosyl, 1 mM EDTA, and 1 mM PMSF) and incubated at 37°C for 10 min. The sample was then subjected to sonication and centrifugation at 13,000 × *g* for 20 min. The final supernatant was harvested as the membrane fraction. The fractions were analyzed by SDS-PAGE and western blot analyses to localize HrcA.

Electromobility shift assay (EMSA)

The EMSA probe was a 150- bp fragment containing the *S. pneumoniae dnaK* CIRCE1 regulatory sequence. The probe was generated by PCR using the primers 5′-ATC TAA TTC ATG GTT ATT TCA GAC-3′ and 5′-ATA CAC CCT TAG AAG AGA ATG TC-3′. One picomole of the PCR-amplified DNA fragment was radiolabeled by adding the probe to reaction

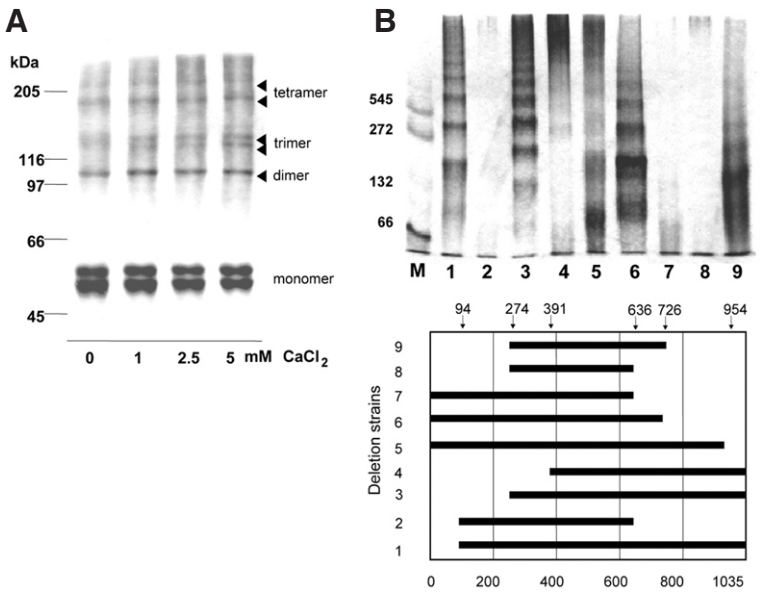


Fig. 1. Reduction-sensitive oligomerization of HrcA. (A) Ca^{2+}-dependent oligomerization of HrcA determined by cross-linking analysis. Four micrograms of HrcA in 20 mM Tris-HCl (pH 7.5) was cross-linked in the presence of 0.1 mM EDAC at the indicated concentrations of Ca^{2+} at 37°C for 30 min. Cross-linked proteins were subject to SDS-PAGE analysis. The oligomeric forms of HrcA are indicated with arrow heads. * The data shown are representative of three independent experiments. (B) Identification of the oligomerization domain. Nested deletion sets of *hrcA* were constructed, overexpressed, and purified (20 mM Tris-Cl, pH 7.6) in *E. coli*. Native- PAGE (6%) analysis (top) and a physical map (bottom) were shown. M, Nondenaturing molecular weight marker. Arrow indicates *hrcA* DNA sequence (base pairs). *The data shown are representative of three independent experiments.

buffer containing 50 mM Tris-HCl, pH 9, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, 50 μCi [γ-^{32}P] ATP and 10 U of T4 polynucleotide kinase. The mixture was then incubated at 37°C for 30 min, applied to a Microcon PCR (Millipore) device, and purified according to the manufacturer's suggested instructions. EMSA was performed using the modified method of Yuan and Wong (1995). The binding reactions (20 μl) contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.2 pmol [γ-^{32}P]-labeled CIRCE fragment, 5 μg sonicated salmon sperm DNA, and 30 μg protein extracted from the *E. coli* strain overproducing HrcA. These reactions were supplemented with 3 pmol of cold CIRCE regulatory element as needed. Following incubation at 25°C for 30 min, samples were immediately loaded onto a 6% native polyacrylamide gel containing 5% glycerol.

Fluorescence spectroscopy and light scattering

The fluorescence emission spectra of 2 μM HrcA in 20 mM Tris-HCl (pH 7.5) buffer containing 10 μM 1,1′-bis(anilino)-4-,4′-bis(naphtalene)-8,8′-disulfonate (bis-ANS) in the presence of Ca^{2+} at 20°C was determined using a F-4010 fluorescence spectrophotometer (Hitachi, Japan). The excitation wavelength was fixed at 397 nm, and emission spectra were scanned from 400 nm to 600 nm. The HrcA amounts cited refer to monomer concentrations. Light scattering of HrcA was measured with the same spectrophotometer using 0.2 μM HrcA in 20 mM Tris-HCl (pH 7.5) at a fixed wavelength of 450 nm for excitation and emission.

Gel filtration analysis

Gel filtration analysis was performed at room temperature using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, Sweden) and an Agilent HPLC system. Aliquots (50 μl) of the purified HrcA and site-directed mutants at a concentration of 0.65 μg/μl were chromatographed on the column pre-equilibrated with 20 mM Tris-HCl (pH 7.5) in the presence or absence of 1 mM $CaCl_2$. The chromatography was performed at a flow rate of 0.5 ml/min at room temperature. The eluted proteins were monitored by absorbance at 280 nm. The protein standards used were as follows: thyroglobulin (bovine), 670,000; gamma globulin (bovine), 158,000; ovalbumin (chicken), 44,000; myoglobin (horse), 17,000; vitamin B-12, 1,350. This experiment was carried out in the cooperative center for research facilities of Sungkyunkwan University.

Statistics

Data is presented either as the mean ± standard deviation (SD) or SEM. One-way analysis of variance (ANOVA) or Student's *t* test was used for statistical analysis. A $P < 0.05$ was considered statistically significant.

RESULTS

Ca^{2+}-dependent oligomerization of HrcA

HrcA is active only in its homodimeric form (Martirani et al., 2001) and binds to CIRCE elements in the presence of Ca^{2+} (Kwon et al, 2005). Thus, the effect of Ca^{2+} on HrcA oligomerization was examined. HrcA was incubated with the cross-linking agent, 1-ethyl-3-[3-dimethyl aminopropyl] carbodiimide (EDAC) at a specific concentration of Ca^{2+}, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The results showed that HrcA was present in an oligomeric form in the absence of Ca^{2+} and became more oligomerized with increasing Ca^{2+} in a concentration dependent manner. HrcA was cross-linked to various intermediates corresponding to monomers (55-kDa), dimers (110-kDa), trimers (165-kDa), and tetramers (220-kDa) (Fig. 1A). However, since crosslinking analysis could not provide the native form of HrcA, gel filtration analysis was performed to determine the native form of HrcA. Purified HrcA was chromatographed on a column equilibrated with 20 mM Tris-HCl (pH 7.5) in the presence or absence of 1 mM $CaCl_2$. HrcA was predominantly present in a tetrameric form in the absence of Ca^{2+} (dimer: 6%, tetramer: 50%, octamer: 31%, hexadecamer: 5%); however, addition of Ca^{2+} caused significant structural transition to the octameric or more highly oligomeric state (dimer: 4%, tetramer: 24% [$P < 0.01$], octamer: 41% [$P < 0.05$], hexadecamer: 20% [$P < 0.05$ compared to in the absence of Ca^{2+}]) (Fig. 2). Since HrcA was more oligomerized in the presence of Ca^{2+}, the oligomerization domain of HrcA was examined by constructing a series of HrcA deletion mutants. N-terminal or C-terminal truncated HrcA was cloned into the pET32a expression vector; however, most of

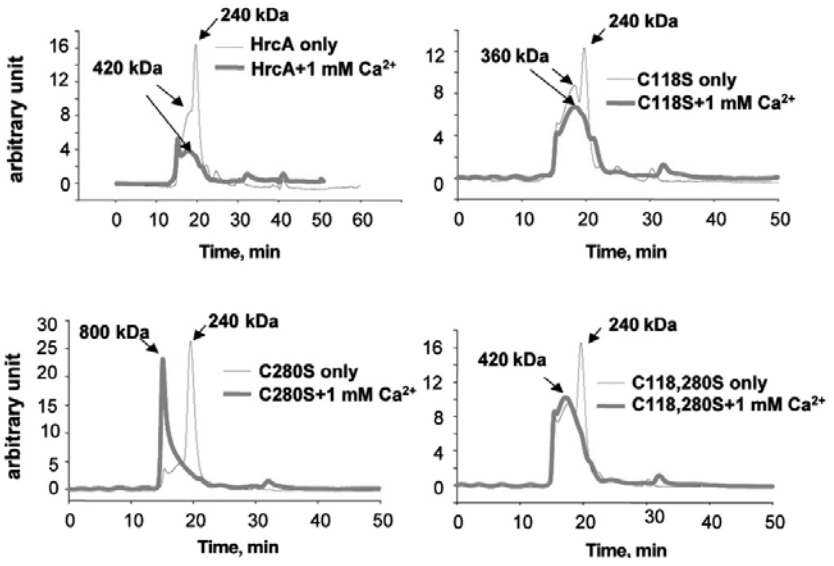


Fig. 2. Ca^{2+}-dependent oligomerization of HrcA determined by gel filtration chromatography. The wild type and site-directed HrcA mutants were equilibrated in 20 mM Tris-HCl (pH 7.5) and chromatographed using a Superdex 200 HR 10/30 column in the presence or absence of 1 mM Ca^{2+}. *The data shown are representative of three independent experiments.

the truncated HrcA was either oligomerized or aggregated (Fig. 1B). Thus, the oligomerization domain of HrcA could not be determined via native PAGE analysis.

Effects of mutations of cysteine residues on HrcA oligomerzation

Cysteine residues have been demonstrated to be involved in protein oligomerization via formation of disulfide bonds (Aruna et al., 2003; Fukuda et al., 1999; Jha et al., 2002) that can be separated with reducing agents, and thus recombinant HrcA was treated with the reducing agent dithiothreitol (DTT) and oligomerization was assessed by SDS-PAGE. The results demonstrated that HrcA was present in an oligomeric form in the absence of reducing agent, whereas it became monomeric in the presence of reducing agent (Fig. 3A), demonstrating that HrcA is sensitive to reduction. Moreover, this result suggests that the cysteine residues of HrcA might be responsible for oligomerization by acting as oxidation-reduction sensors. *S. pneumoniae* HrcA has two cysteine residues at positions 118 and 280 that are not conserved in other organisms. Therefore, theses residues were replaced with serine using site-directed mutagenesis to produce the single mutants C118S and C280S, and the double mutant C118 280S. SDS-PAGE analysis revealed that in the absence of a reducing agent, the double mutant HrcA (C118, 280S) was not oligomerized; however, all the other forms of HrcA were oligomerized (Fig. 3B). To corroborate HrcA's response to oxidation-reduction, HrcA was reduced by DTT followed by exposure to various concentrations of hydrogen peroxide. After exposure to H_2O_2, the monomeric structure of the HrcA became oligomeric in an H_2O_2 dependant manner, (Fig. 3C) demonstrating that that structure of reduced HrcA can be reverted to its oligomeric form by oxidation. In the absence of Ca^{2+}, both the C118S and C280S HrcA mutants were dominantly present in the tetrameric form, whereas they were present in the hexameric to hexadecameric form in the presence of Ca^{2+}. In the presence of Ca^{2+}, the C118S HrcA mutant was present in the hexameric to heptameric form, while the C280S HrcA mutant was present in the tetradecameric to hexadecameric form (Fig. 2). Thus, the C118S mutant existed in a less oligomerized form and the C280S mutant was more oligomerized in the presence of Ca^{2+} than wild type HrcA (Fig. 2). The C118 280S double mutant exhibited an oligomerization profile similar to that of wild type HrcA.

Effects of cysteine residue mutations on HrcA aggregation and exposure of hydrophobic surfaces

Since the cysteine residues in HrcA appeared to affect its oligomerization state in the presence of Ca^{2+}, aggregation patterns of the wild type and the mutant forms of HrcA were examined after supplementation of calcium. As shown in Fig. 4, both the wild type and the mutants did not form aggregates in the absence of calcium. However, in the presence of calcium, the C118S mutant HrcA exhibited milder aggregation compared to the wild type, whereas the C280S mutant exhibited more severe aggregation compared to the wild type. Interestingly, the HrcA C118 280S double mutant exhibited an aggregation pattern similar to that of the wild type HrcA, providing a good agreement with the oligomerization results (Fig. 4).

Since the oligomerization state of the wild type and the mutant HrcA was affected by the presence of cysteine, the effect of cysteine on the exposure of hydrophobic surfaces was examined in the presence or absence of calcium using the bis-ANS fluorescence method. As shown in Fig. 5 and Table 1, exposure of the hydrophobic surface in all of the mutants was increased after calcium supplementation. The largest increase was observed in the double mutant C118 280S in the presence of calcium. In contrast, only a slight increase was observed for mutant C280S in the presence of calcium.

Effects of cysteine residue mutations on HrcA binding to CIRCE

To investigate the ability of HrcA to bind to CIRCE elements, the effects of cysteine residue mutations on HrcA binding to CIRCE were examined using electromobility shift assays (EMSA) assays. Consistent with the previous report (Kwon et al., 2005), binding of HrcA to CIRCE was low. The percentage of band-shift in the C118S mutant was 8.3%, which was the lowest of all HrcA forms, including wild type, and was significantly different from that of wild type HrcA ($p < 0.05$). The shift percentage of the C280S mutant was 12%, which was the second lowest. Conversely, the double mutant C118 280S had similar or even slightly higher efficiency in binding to CIRCE elements compared with that of the wild type HrcA (Fig. 6).

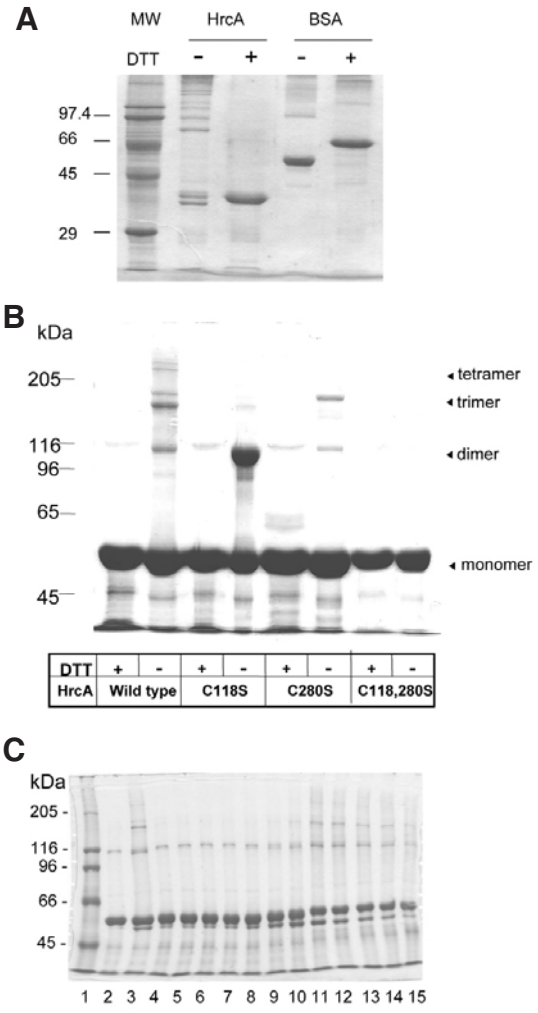


Fig. 3. Oligomerization of HrcA. (A) Monomerization of HrcA by reduction. Denatured (6 M urea-treated) and purified HrcA (5 μg) was incubated in the presence or absence of the reducing agent dithiothreitol (100 mM) at 25°C for 20 min and subjected to cross-linking followed by SDS-PAGE (10%). Bovine serum albumin (BSA) was used as a control. (B) Disulfide bond-mediated HrcA oligomerization in the absence of Ca^{2+}. Wild type and mutant HrcAs were incubated at room temperature in the presence or absence of 10 mM DTT and subjected to cross-linking followed by SDS-PAGE without β-mercaptoethanol. (C) Oxidation-mediated oligomerization. Purified HrcA was incubated with 5 mM DTT for 30 min at room temperature, followed by an exchange with buffer containing various concentrations of hydrogen peroxide for 5 min at room temperature. The samples were then subjected to cross-linking followed by SDS-PAGE analysis. Lane 1, molecular weight marker; lane 2, HrcA treated with DTT; lane 3, HrcA without treatment; lanes 4-15, HrcA + DTT + H_2O_2 which contains H_2O_2 from 50 μM (lane 4), 100 μM (lane 5), 200 μM (lane 6), 400 μM (lane 7), 1 mM (lane 8), 5 mM (lane 9), 10 mM (lane 10), 50 mM (lane 11), 100 mM (lane 12), 150 mM (lane 13), 200 mM (lane 14), lane 250 mM (lane 15). *The data shown are representative of three independent experiments.

Since HrcA is oligomerized under a normal oxidized state but becomes monomeric after reduction, we attempted to determine CIRCE element binding in the reduced state, but were not successful due to immediate aggregation.

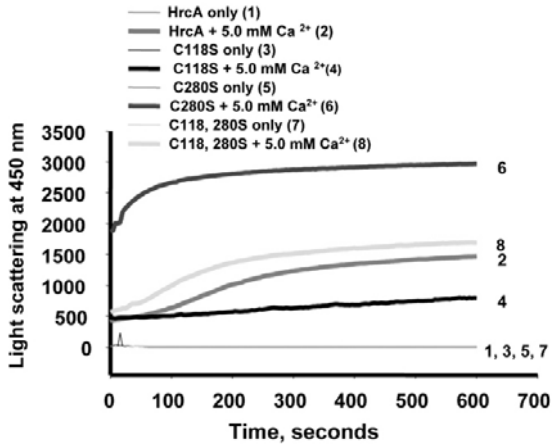


Fig. 4. Ca^{2+}-dependent aggregation of HrcA. Ca^{2+}-dependent agregation of the mutated HrcA was determined by light scattering. The wild type and mutant HrcAs were diluted to a final concentration of 0.2 μM in 20 mM Tris-HCl (pH 7.5) in the absence (lines 1, 3, 5, and 7) or presence (lines 2, 4, 6, and 8) of Ca^{2+}. The increase in light scattering at 450 nm was examined as a function of the incubation time. *The data shown are representative of three independent experiments.

Localization of HrcA to the membrane

HrcA was recently identified as a membrane-associated protein in *H. pylori* (Roncarati et al., 2007). In our study, pneumococcal HrcA was found to contain two predicted hydrophobic transmembrane domains: from the 134-151th residue and from the 230-249th amino acid residue (www.ch.embnet.org/software/TMPRED). Moreover, pneumococcal HrcA was sensitive to reduction. These findings prompted us to examine the possible localization of pneumococcal HrcA in the membrane. We attempted to fractionate subcellular proteins of *S. pneumoniae* and to identify localization of HrcA after heat shock, but failed to do so because the HrcA complex is unstable at high temperatures (Watanabe et al., 2001). Further, inactive HrcA recovers binding activity in the presence of GroEL (Martirani et al., 2001; Mogk et al., 1998), and thus the failure of HrcA fractionation and subsequent detection was likely due to the unstable nature of HrcA at high temperatures. Therefore, to examine localization of HrcA, HrcA without a histidine tag was overexpressed in *E. coli* and subcellular proteins were processed into periplasm, membrane, and cytosolic fractions. Next, the fractionated proteins were subjected to SDS-PAGE and immunoblot analysis. HrcA was detected in both the cytosolic (52% of the total proteins) and membrane (90% of the total proteins) fractions (Figs. 7A and 7B).

Membrane proteins can be either integral or associated in terms of their interaction with the cell membrane. Integral membrane proteins are directly anchored to the lipid bilayer through one or more transmembrane regions, whereas membrane-associated proteins contain neither transmembrane domains nor lipid modifications, and are instead anchored to the lipid bilayer through noncovalent interactions with integral membrane proteins and are can be removed from the membrane by mechanical forces such as washing (Schindler et al., 2006; Zhao et al., 2004). To determine whether HrcA is a membrane-associated protein or a transmembrane protein, the membrane fraction was subjected to washing with 6 M urea or 0.1 M Na_2CO_3, pH 11.5. Neither the chaotropic detergent nor the high-pH wash (0.1 M Na_2CO_3, pH 11.5) decreased the

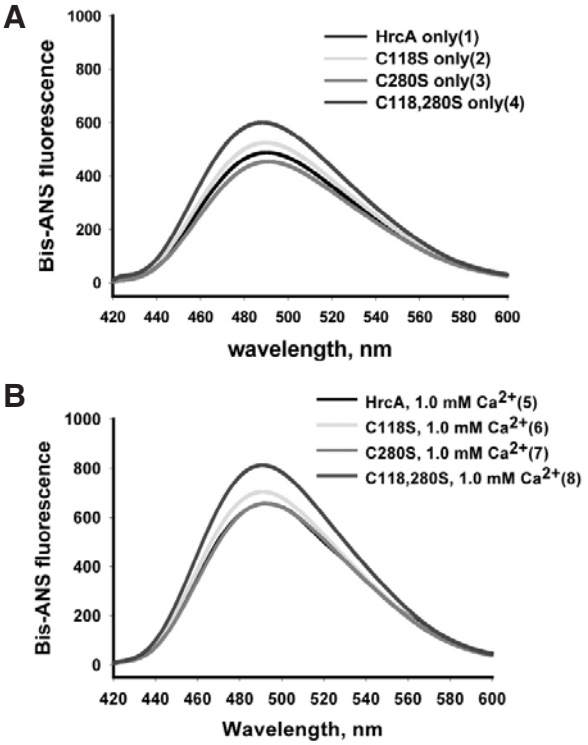
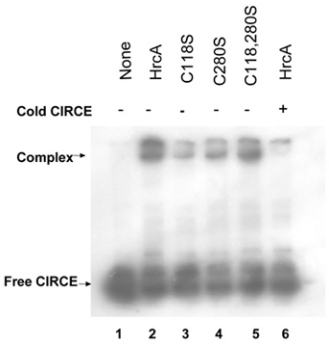


Fig. 5. Ca^{2+}-dependent modulation of bis-ANS fluorescence emission spectra of mutant HrcA. The wild type and mutant HrcAs were incubated with Bis-ANS in the absence (lines 1, 2, 3, and 4) or presence (lines 5, 6, 7, and 8) of Ca^{2+} ion. Bis-ANS binding to the wild type and mutant HrcA was enhanced in the presence of Ca^{2+}, indicating the exposure of hydrophobic surfaces. The fluorescence data shown are in arbitrary units. *The data shown are representative of three independent experiments.



Protein	Wild type	C118S	C280S	C118, 280S
Shifted band, %	18.8 ± 4.8	8.3 ± 6.9*	12.0 ± 9.4	21.1 ± 11.4

Fig. 6. Requirement of cysteine residues for formation of the CIRCE-HrcA complex *in vitro*. Electromobility shift assays were performed by incubating *E. coli* extracts overproducing HrcA (30 μg) with a [γ-^{32}P]-labeled CIRCE fragment at 25°C for 30 min and then loading the samples onto a 6% native polyacrylamide gel, followed by autoradiography. Lane 1, CIRCE probe alone; lanes 2-5, protein extract from *E. coli* strain overproducing the wild type or the mutant HrcA; lane 6, protein extract from *E. coli* strain overproducing the wild type HrcA in the presence of cold CIRCE. Results from three independent experiments are presented as the mean percentage of shifted band ± SD (* P <0.05). A P value of <0.05 is considered statistically significant.

amount of HrcA amount in the membrane fraction, suggesting that HrcA is an integral membrane protein (Figs. 7C and 7D).

DISCUSSION

The majority of HrcA protein is inactive, with only small fraction able to bind CIRCE elements, as determined through EMSA assays (Minder et al., 2000; Mogk et al., 1997). In addition, we found that purified *S. pneumoniae* HrcA did not bind CIRCE in the presence of GroEL or DnaK either alone or in combination, although all components of either the DnaK chaperone system (DnaK, GrpE, and DnaJ) or GroE chaperone system (GroEL and GroES) were not included for our EMSA assays. Therefore, *E. coli* cell lysates overproducing HrcA were used for *in vitro* protein-DNA interaction analysis, which show that HrcA is able to bind CIRCE elements in a Ca^{2+} dependent manner (Kwon et al., 2005).

In this study, *S. pneumoniae* HrcA was found to be present as higher order oligomers, such as tetramers and octamers. Moreover, *S. pneumoniae* HrcA contains two cysteine residues. In contrast, *Streptococcus thermophilus* HrcA forms a dimer (Martirani et al., 2001), and *S. aureus* HrcA binds to CIRCE elements as a dimer with a twofold axis of symmetry due to the palindromic nature of the HrcA binding site (Chastanet et al., 2003). Thus *S. pneumoniae* HrcA appears to have a unique ability to form higher order oligomers.

In the presence of Ca^{2+}, *S. pneumoniae* HrcA became more oligomerized, forming tetramers through octamers. Previously it was shown that in the presence of Ca^{2+}, *S. pneumoniae* HrcA becomes partially unfolded and thermally destabilized (Kwon et al., 2005). Similar to pneumococcal HrcA, thermostability of heat shock protein 90 (HSP90) in brain is strongly decreased by Ca^{2+}, although HSP90 is not a calcium binding protein. Moreover, Ca^{2+}-dependent oligomerization of HSP90 at lower temperatures is responsible for its ability to bind substrate proteins and prevent their irreversible aggregation (Garnier et al., 1998). Since unfolded proteins possess a high degree of exposed hydrophobic surfaces, it has been proposed that the hydrophobic interactions as well as flexible and expanded conformations are the driving forces behind the association of misfolded proteins with the GroEL chaperone (Lin et al., 1995; Okazaki et al., 1994). Consistent with these observations, we found that in the presence of Ca^{2+}, exposure of hydrophobic surfaces on HrcA was greatly increased, as was oligomerization of HrcA and refolding by GroEL as well. Finally, these processes led to increased binding of HrcA to CIRCE elements.

Cross-linking followed by SDS-PAGE analysis of recombinant HrcA revealed that HrcA was present in its oligomeric form in the absence of a reducing agent. To determine the role of cysteine residues on oligomerization, the two cysteine residues were replaced by serines. SDS-PAGE analysis without reducing agent indicated that that double mutant C118 280S was not oligomerized (Fig. 3B), whereas gel filtration analysis (Fig. 2) showed that both wild type HrcA and the other mutants forms of HrcA were oligomerized. This incompatibility between results suggests that HrcA oligomerization could be mediated by two domains, where one domain is mediated by cysteine residues which are SDS-resistant, and the other by an unknown domain that is SDS-sensitive. Another interesting result was that the N-terminal cysteine residue appeared to be involved in recruiting HrcA into a more highly oligomerized state, whereas the C-terminal cysteine residue promoted less oligomeric forms in the

Table 1. Bis-ANS fluorescence emission spectra of mutant HrcA

HrcA	Wild type	C118S	C280S	C118,280S
buffer only	505.4 ± 20.1[a] (1.0)[c]	520.0 ± 4.4 (1.03)***[d]	456.8 ± 20.9 (0.9)**[d]	597.6 ± 8.3 (1.18)***[d]
Buffer + 1.0 mM Ca^{2+} (***)[b]	661.9 ± 27.5 (1.31)	712.3 ± 9.2 (1.41)**[e]	651.2 ± 14.1 (1.29)	811.6 ± 6.9 (1.61)***[e]

[a]Arbitrary unit that indicates the extent of hydrophic surface exposure (mean ± SD)
[b]The hydrophobic surface of wild type and all HrcA mutants was more exposed in the presence, rather than in the absence, of 1 mM Ca^{2+} ($p < 0.001$).
[c]Fold increase is proportional to the wild type HrcA in the absence of calcium.
[d]Mutant HrcA was compared with the wild type HrcA in the absence of calcium.
[e]Mutant HrcA was compared with the wild type HrcA in the presence of calcium (*, $p < 0.05$), (**, $p < 0.01$), (***, $p < 0.001$).

presence of Ca^{2+}. Thus, the two cysteine residues may function as checks to one another to determine whether HrcA forms a functional oligomer complex. The results of aggregation analysis were in accordance with the oligomerization analysis findings; however, the bis-ANS binding (analysis of exposure of hydrophobic surfaces) was inversely proportional (Figs. 4 and 5; Table 1) to the oligomerization findings. Specifically, the C118S mutant induced lower oligomerization, exhibited less aggregation, and had a slightly higher affinity for bis-ANS, whereas the C280S mutant induced higher oligomerization, exhibited significantly increased aggregation, and had a slightly lower affinity for bis-ANS in the presence of Ca^{2+}. These results suggest that as oligomerization is induced by Ca^{2+}, aggregation is accelerated through the interaction of hydrophobic surfaces, thus resulting in low bis-ANS binding. The HrcA double mutant C118 280S exhibited an oligomerization and aggregation profile similar to that of wild type HrcA. Unexpectedly, bis-ANS binding to the double mutant was significantly higher than that of the wild type in the absence or presence of Ca^{2+}. Consistent with this paradoxical finding, HrcA from the thermophile *Thermotoga maritime* has been shown to have an inserted dimerization domain in the site for monomer-monomer interactions (Liu et al., 2005). However, this conformation does not appear to be compatible with DNA binding and may represent an inactive form of HrcA (Liu et al., 2005). Therefore, our findings for the double mutant might be due to it being in an inactive form, although the mechanism of action of this mutant should be clarified in future studies.

S. pneumoniae HrcA contains a helix-turn-helix motif near the N-terminal, and thus the N-terminal portion of HrcA may be involved in DNA binding. Indeed, the N-terminal portion of *Bacillus subtilis* HrcA, which also contains a helix-turn-helix motif, has been shown to be involved in DNA binding (Wiegert, 2003). Although the DNA binding motif of *S. pneumoniae* HrcA does not contain cysteine residues, it may be possible that the cysteine residues influence CIRCE element binding through control of the oligomerization state. Consistent with this hypothesis, our *in vitro* EMSA assays showed that the C118S mutant, which induces a slightly less oligomerized form of HrcA, binds to the CIRCE element less efficiently compared with the wild type or C280S mutant, suggesting that HrcA cysteine residues contribute to CIRCE binding, at least to some extent *in vitro*, through control of oligomerization.

Heat shock proteins are induced by stresses and are therefore resistant to these harmful conditions. Pneumococci regularly experience different levels of oxido-reductive conditions during invasion of the blood and meninges from the nasopharynx. In addition, Ca^{2+} pretreatment represses transcription of the *dnaK* and *groEL* operons as well as pneumococcal growth under heat shock conditions (Kwon et al., 2005). Despite these findings, however, the effect of Ca^{2+} on HrcA protein was unknown prior to this study. Here, we demonstrated for the first time that HrcA activity can be modulated by oxidation-reduction and that pneumococcal HrcA binding to CIRCE was inhibited by the C118S mutation, thereby suggesting that the cysteine residues of HrcA are responsible for oligomerization and CIRCE binding. Further, more precise knowledge of the stress regulation mechanism of human pathogens is important, and further study of these processes in *S. pneumoniae* should be very useful for prevention of infection by this pathogen and for treatment of *S. pneumoniae* infections.

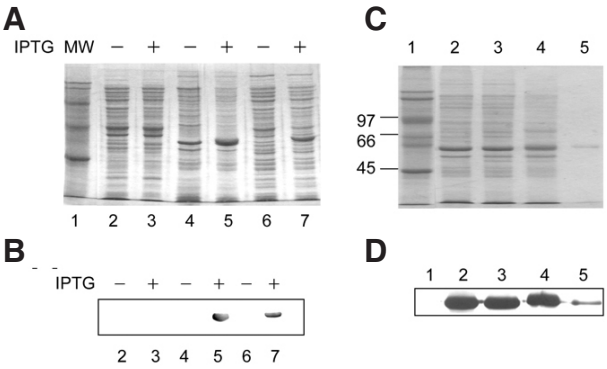


Fig. 7. Localization of HrcA. (A, B) Identification of HrcA localization in *E. coli*. HrcA without a his-tag was overexpressed in *E. coli* and the cellular proteins were processed into periplasmic (lanes 2, 3), membrane (lanes 4, 5) and cytosolic (lanes 6, 7) fractions and were subjected to SDS-PAGE (A) and western blot analysis (B). (C, D) Identification of HrcA as a transmembrane protein. Isolated membrane fraction (10 μg) was incubated in 6 M urea or 0.1 M Na_2CO_3 (pH 11.5) at 4°C for 40 min and subjected to SDS-PAGE (C) and western blot analysis (D). Lane 1, molecular marker; lane 2, membrane fraction; lane 3, supplemented with 6 M urea; lane 4, supplemented with 0.1 M Na_2CO_3 (pH 11.5); lane 5, purified HrcA. *The data shown are representative of three independent experiments.

The physical state of the cellular membrane is linked to thermal stress, and thermal stress can be sensed and transmitted to activate HSP genes (Horváth et al., 1998; Vigh et al., 1998). Moreover, HSP expression can be induced by even subtle abnormalities in the specific local nonbilayer microdomains of membranes (Horváth et al., 1998; Vigh et al., 2007). Because molecular chaperones of the Hsp70 and Hsp100 family are associated with the translocation complex, and are able to interact with translocating precursors (Akita et al., 1997; Lim et al., 2001; Liu et al., 2001; McClellan et al., 1998; Nielson et al., 1997), it is likely that HSP expression can be modulated by the physical state of the membrane, such as hydrophobicity and fluidity. Furthermore, *Helicobacter pylori* HrcA has been found in the inner membrane (Roncarati et al., 2007). Here, we demonstrated that the hydrophobicity of HrcA was increased in the presence of Ca^{2+}. Likewise, the location of HrcA in the membrane suggests that the hydrophobic parts of HrcA may be

involved in membrane fluidity upon thermal stress, transmitting signals to modulate *dnaK* and *groE* operons. However, the mechanism underlying this proposed pathway remains unknown.

Normal blood Ca^{2+} levels in adults are between 2.2 and 2.7 mM (http://www.webmd.com/a-to-z-guides/calcium-ca-in-blood). At these physiological Ca^{2+} levels, HrcA inhibits expression of the CIRCE regulon as well as thermotolerance (Kwon et al., 2005). In contrast, *dnaK* and *groEL* genes were induced in blood (Oggioni et al., 2006) suggesting that other factors are also involved in regulation of the CIRCE regulon. Moreover, *dnaK* and *groE* operons are dually regulated by CtsR and HrcA in *S. pneumoniae* (Chastanet et al., 2003). Similarly, *dnaK* and *groE* operons are dually regulated by σ^B and HrcA in *Listeria monocytogenes* (Hu et al., 2007), or by HrcA and HspR in *Campylobacter jejuni* (Stintzi et al., 2005). Therefore, the *hrcA* mutation alone did not affect invasion into the host cells nor colonization in these organisms (Hu et al., 2007; Stintzi et al., 2005). In the same manner, the C118S *hrcA* mutation in *S. pneumoniae* would not affect virulence although the mutant C118S HrcA has reduced binding to CIRCE elements and is less oligomerized in the presence of Ca^{2+}. Further studies are required to verify this hypothesis.

Collectively, our data indicate that HrcA acts as a reduction-oxidation sensor via a Ca^{2+}-induced conformational rearrangement resulting in oligomerization, which subsequently leads to exposure of hydrophobic surfaces and promotes binding to CIRCE elements and repression of the CIRCE regulon. Lastly, the cysteine residues of HrcA appeared to play important roles in mediating proper oligomerization, oxidation sensing, and CIRCE element binding.

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