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Chlamydial GroEL Autoregulates Its Own Expression through Direct Interactions with the HrcA Repressor Protein

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In the pathogenic bacterium *Chlamydia trachomatis*, a transcriptional repressor, HrcA, regulates the major heat shock operons, *dnaK* and *groE*. Cellular stress causes a transient increase in transcription of these heat shock operons through relief of HrcA-mediated repression, but the pathway leading to derepression is unclear. Elevated temperature alone is not sufficient, and it is hypothesized that additional chlamydial factors play a role. We used DNA affinity chromatography to purify proteins that interact with HrcA in vivo and identified a higher-order complex consisting of HrcA, GroEL, and GroES. This endogenous HrcA complex migrated differently than recombinant HrcA, but the complex could be disrupted, releasing native HrcA that resembled recombinant HrcA. In in vitro assays, GroEL increased the ability of HrcA to bind to the CIRCE operator and to repress transcription. Other chlamydial heat shock proteins, including the two additional GroEL paralogs present in all chlamydial species, did not modulate HrcA activity.

The heat shock response is a transient increase in the synthesis of heat shock proteins in response to cellular stressors, such as elevated temperature. These heat shock proteins are molecular chaperones and proteases that play an important role in preventing the accumulation of misfolded proteins. Increased synthesis of the heat shock proteins in response to stress occurs mainly at the transcriptional level, although other mechanisms are utilized by different organisms to achieve the same result (47). Many bacteria control the transcription of heat shock genes with a negative regulator, HrcA, and it is the relief of repression in response to heat shock that leads to increased expression levels (26, 38). While it is clear that derepression involves dissociation of HrcA from its cognate operator, CIRCE, the molecular details are unclear, and two competing models have been proposed. The models differ in whether HrcA-CIRCE binding is disrupted by the direct effects of elevated temperature (22, 31, 46) or indirectly through the actions of one or more factors that sense cellular stress. Particular attention has focused on the chaperonin GroE, which is itself regulated by HrcA (23, 48).

We are interested in the regulation of heat shock proteins in *Chlamydia* because of their long-recognized association with pathogenesis. The chlamydial heat shock proteins, GroEL (Hsp60) and GroES (Hsp10), have been implicated in the chronic inflammatory process that is the hallmark of chlamydial infections and which leads to tissue damage and scarring (20, 27, 35, 36). Chlamydial GroEL has been serologically associated with the various manifestations of chlamydial infection, including trachoma (27) and pelvic inflammatory disease (28, 44), and the sequelae of infection, such as elevated risks of ectopic pregnancy and tubal infertility in women (1, 6). GroEL from *Chlamydia pneumoniae* has been detected in atherosclerotic plaques (18), and serum antibodies to GroEL correlate with the ability for *C. pneumoniae* to be detected in plaques (10), supporting a role for chlamydial infection in the pathogenesis of atherosclerotic heart disease (13).

In this report, we examine how expression of the heat shock genes is regulated in *Chlamydia* through interactions between the HrcA repressor and GroEL and GroES, which together make up the GroE chaperone complex. We have previously shown that HrcA regulates both the *dnaK* and *groE* heat shock operons in *Chlamydia trachomatis* through binding to the CIRCE operator (50, 51), but increased temperature within physiologic limits could not affect HrcA-mediated repression (51). We now show that HrcA, GroEL, and GroES can be isolated together from a chlamydial lysate as a complex bound to CIRCE. GroEL by itself was able to increase both the binding affinity of HrcA for CIRCE and the repression of the heat shock promoters by HrcA. These findings provide experimental support for key elements of the GroE titration model (23, 26), which proposes twin roles of GroE in the response to stress as both a molecular chaperone and a regulator of heat shock gene expression.

MATERIALS AND METHODS

DNA affinity purification of native HrcA. DNA affinity magnetic beads were generated by attaching biotinylated DNA containing the *dnaK* CIRCE operator sequence of *C. trachomatis* serovar L2 to streptavidin-conjugated beads by following the method of Gabrielsen and Huet (11). The *C. trachomatis* serovar MoPn *dnaK* CIRCE DNA fragment was produced by digesting the plasmids pMT1173 or pMT1122 (Table 1) with the restriction enzymes EcoRI, ApaI, and SacI. The DNA fragments with 5′ overhangs were biotinylated with a fill-in reaction by using the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of biotin-14-dATP by following the manufacturer's directions (Invitrogen, Carlsbad, CA). Complete biotinylation of the 456-bp target fragment was verified by incubation with streptavidin at 37°C for 10 min followed by electrophoresis on a 1% agarose gel. Unincorporated deoxynucleoside triphos-

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TABLE 1. Plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pRSET-C	pUC-derived T7 promoter-based expression plasmid	Invitrogen
pMT1133	pRSET-C expressing *C. trachomatis* serovar L2 *hrcA*	50
pMT1132	pRSET-C expressing *C. trachomatis* serovar D *groEL1*	This study
pMT1209	pRSET-C expressing *C. trachomatis* serovar D *groES*	This study
pMT1206	pRSET-C expressing *C. trachomatis* serovar D *groEL2*	This study
pMT1207	pRSET-C expressing *C. trachomatis* serovar D *groEL3*	This study
pMT1205	pRSET-C expressing *C. trachomatis* serovar D *dnaK*	This study
pGEM-7Zf(+)	PGEM-3Zf(+)-derived cloning vector containing f1 origin of replication	Promega
pMT1134	pGEM-7Zf(+) containing *C. trachomatis* serovar L2 *dnaK* CIRCE (−98 to −2)	50
pMT1173	pGEM-7Zf(+) containing multimerized *C. trachomatis* serovar L2 *dnaK* CIRCE (−98 to −2)	This study
pMT1122	pGEM-7Zf(+) containing *C. trachomatis* serovar L2 *groE* upstream region without CIRCE	50
pMT1175	pGEM-7Zf(+) containing *C. trachomatis* serovar D *groE* CIRCE (−98 to −2)	51
pMT1125	pGEM-7Zf(+)-based promoterless, G-less cassette transcription template	50
pMT1138	pMT1125 containing *Chlamydia muridarum* strain Nigg *dnaK* promoter (−135 to +5)	50
pMT1178	pMT1125 containing *C. trachomatis* serovar D *groE* promoter (−135 to +5)	51
E. coli strains		
BL21(DE3)	Bacteriophage T7 polymerase inducible strain for protein expression	Invitrogen
XL1-Blue	*recA* strain for plasmid propagation	Stratagene
JM109 (pGP57)	Transformed strain constitutively expressing *C. psittaci* GroEL and GroES	25

phates were removed using DNA minispin columns (Roche, Indianapolis, IN), and the DNA was diluted 1:10 in buffer TEN (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl). Biotinylated DNA was incubated with washed Dynabead M-280 streptavidin-conjugated magnetic beads (Dynal, Brown Deer, WI) for 15 min at room temperature. In all experiments, beads were pelleted using the Dynal Magnetic Bead Concentrator for 2 min. Beads were washed three times with buffer TEN to remove unbound DNA. When not in use, beads were stored in buffer TEN at 4°C.

To obtain chlamydial reticulate body lysates, 7.5×10^8 murine L929 host cells grown in suspension were infected with *C. trachomatis* serovar L2 at a multiplicity of infection of 3. Infected cells were harvested at 28 h postinfection, partially purified, and lysed as previously described (41). The reticulate body lysate was diluted in buffer I (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 7.5% glycerol) to an NaCl concentration of 150 mM, applied to DNA-conjugated magnetic beads preequilibrated with the same buffer, and incubated for 20 min at 4°C with gentle rocking. Beads were washed twice with buffer I [200 mM NaCl] to remove nonspecifically bound proteins. Proteins bound to the beads were eluted in two exchanges of buffer I [600 mM NaCl]. Eluted proteins were dialyzed against storage buffer (10 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 100 μM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaCl, 30% glycerol) overnight and again for 4 h. For some experiments, the CIRCE-bead eluate was denatured by the addition of urea to a final concentration of 6 M. The urea was removed through overnight dialysis against storage buffer.

Mass spectrometry. Coomassie-stained protein bands were excised for in-gel protease digestions. Briefly, each gel band was excised and washed in 100 mM NH_4HCO_3. Proteins were reduced by incubation in 25 mM dithiothreitol–100 mM NH_4HCO_3 for 15 min at 60°C. After cooling to room temperature, proteins were alkylated by incubation in 75 mM iodoacetamide–100 mM NH_4HCO_3 for 15 min in the dark. Gel bands were then washed with 50% acetonitrile–100 mM NH_4HCO_3 for 20 min and divided into three equal slices. Each slice was cut into 1-mm^3 pieces and dehydrated in 100% acetonitrile for 15 min. After the solvent was removed, the gel pieces were dried in a Speed-Vac (ThermoElectron, Waltham, MA). The three aliquots of dried gel pieces were rehydrated separately in the following solutions: (i) 0.5 μg modified trypsin (Promega, Madison, WI) in 25 mM NH_4HCO_3, (ii) 0.5 μg elastase (Boehringer Mannheim, Indianapolis, IN) in 25 mM NH_4HCO_3, and (iii) 0.5 μg subtilisin in 25 mM NH_4HCO_3. Additional buffer without enzyme was added to cover gel pieces, and the samples were incubated at 37°C for 4 h (subtilisin) and 12 h (elastase and trypsin). Peptides were extracted from the gel pieces with 100 μl 60% acetonitrile–0.1% trifluoroacetic acid for 20 min at 28°C on a Thermomixer (Eppendorf, Hamburg, Germany) set at 1,400 rpm. The trypsin, elastase, and subtilisin extracts were pooled and dried by using a Speed-Vac.

The dried peptide sample was resuspended in 20 μl 5% formic acid and pressure loaded onto a triphasic microcapillary column constructed from 100-μm intradermally fused silica capillary tubing pulled to a 5-μm intradermal tip using a P-2000 CO_2 laser puller (Sutter Instruments, Novato, CA) and packed with 7 cm of 5-μm Aqua C18 material (Phenomenex, Torrance, CA) and 4 cm of 5-μm PartiSphere SCX (strong cation exchanger) (Whatman, Clifton, NJ), followed by 2 cm of Aqua C18. The column was then placed in-line with an Agilent 1100 quaternary high-pressure liquid chromatography (HPLC) pump (Palo Alto, CA) and analyzed using a 12-step separation described previously (52). The HPLC pump was operated at a flow rate of 100 μl/min and split to obtain flow through the column of ~100 to 200 nl/min. As peptides eluted from the microcapillary column, they were electrosprayed into an LCQ-Deca mass spectrometer (ThermoElectron) with the application of a 2-kV spray voltage applied distally to the waste of the HPLC split. A cycle of one full-scan mass spectrum (400 to 1,400 *m/z*), followed by three data-dependent tandem mass spectrometry (MS/MS) spectra at a 35% normalized collision energy, was repeated continuously throughout each step of the multidimensional separation. Application of the mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcaliber data system (ThermoElectron). Tandem mass spectra were analyzed by using the following software analysis protocol. The 2to3 analysis is a software protocol for determining the charge state (+2 or + 3) of multiply charged peptide spectra and deleted poor quality spectra. Each MS/MS spectrum after 2to3 was used to search a protein database containing *Chlamydia*, human, mouse, and rat sequences concatenated into a single fasta file by using Sequest (8). All searches were performed without any enzyme specificity. The program DTASelect was used to filter peptide sequences from +1-, +2-, and +3-charged peptide precursors with normalized Sequest XCorr scores of >0.3 (21) and ΔCn scores of >0.1 to assemble peptide sequences into proteins and to remove redundant protein sequences (40).

Protein electrophoresis and immunodetection. Protein samples for gel electrophoresis were suspended in reducing sample buffer (50 mM Tris-HCl [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 0.1% bromphenol blue) and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (2). Proteins were then transferred to nitrocellulose by use of Semi-Dry Trans-Blot (Bio-Rad, Hercules, CA) and blocked in 5% dry milk–TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 30 min at room temperature. The following primary antibodies were obtained from the following sources: α-HrcA antibody was described previously (50); α-GroEL antibody (A57-B9) (53) was a generous gift of R. P. Morrison, University of Alabama, Birmingham; α-MOMP antibody (E4) (29) was a generous gift of E. M. Peterson, University of California, Irvine, Calif.; α-GroES antibody (M1.4) (19) was obtained from Stressgen Bioreagents (Victoria, British Columbia, Canada); α-DnaK antibody (18.1) (5) was a generous gift of S. Birkelund, University of Aarhus, Aarhus, Denmark; and α-GroEL2 antibody (K5) was a generous gift of R. Brunham, University of British Columbia Centre for Disease Control, Vancouver, British Columbia, Canada. Rabbit polyclonal α-RpoA and α-RpoD antibodies were generated following the protocol used for the poly-

TABLE 2. Relevant loci, genes, and proteins

Locus	Gene	Protein
CT394	*hrcA*	HrcA
CT110	*groEL*	GroEL/HSP60
CT111	*groES*	GroES/HSP10
CT396	*dnaK*	DnaK/HSP70
CT322	*tufA*	EF-Tu
CT681	*ompA*	MOMP
CT236	*acpP*	AcpP

clonal α-HrcA antibody (50). Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse immunoglobulin polyclonal antibodies) were obtained from MP Biomedical (Irvine, CA). α-HrcA and α-RpoA polyclonal antibodies were purified by using protein G-conjugated magnetic beads (Dynal) following manufacturer's instructions. Immunoreactivity was visualized by incubating the blot in enhanced chemiluminescence solution (100 mM Tris-HCl [pH 8.5], 450 μM *p*-coumaric acid, 1.25 mM 3-aminophthalhydrazide, 0.015% hydrogen peroxide) for 2 min at room temperature followed by exposure to X-ray film.

Overexpression and purification of recombinant proteins. His_6-tagged recombinant proteins were overexpressed in freshly transformed *E. coli* BL21(DE3) and purified as previously described (50). Coexpressed recombinant GroEL and GroES of *Chlamydia psittaci* were purified following a previously published protocol (30).

EMSA. Standard electrophoretic mobility shift assay (EMSA) reactions were performed as previously described (51).

For gel extraction experiments, multiple lanes were excised from EMSA gels following electrophoresis and sectioned into 10 fractions corresponding to different gel mobility. Gel slices were homogenized through repeated maceration with 1-ml syringes, following the method of Scheer and Ryan (37). Gel slices were then soaked in elution buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, 150 mM NaCl) overnight at 4°C. Following centrifugation to remove polyacrylamide fragments, proteins were precipitated from supernatants using 0.25 volumes of TCA-DOC solution (100% trichloroacetic acid, 4 mg/ml Na deoxycholate). Pellets were washed twice in cold acetone and dried under vacuum. Proteins were resuspended in sample buffer and analyzed by SDS-PAGE. In parallel, sectioned EMSA gel lanes were analyzed by liquid scintillation counting to determine which samples contained ^{32}P-labeled DNA fragments.

Purification of C. trachomatis RNA polymerase. RNA polymerase was partially purified from *C. trachomatis* serovar L2 at 24 h postinfection by heparin-agarose chromatography as previously described (41).

In vitro transcription. Chlamydial in vitro transcription reactions with recombinant proteins were performed as previously described (51).

RESULTS

Endogenous HrcA isolated from a chlamydial lysate copurifies with other proteins. To determine if additional factors modulate transcriptional repression by HrcA, we searched for proteins that can interact with the HrcA-CIRCE complex. In the absence of genetic transformation or directed in vivo mutagenesis techniques in *Chlamydia*, we utilized a biochemical approach to isolate endogenous chlamydial HrcA on the basis of specific binding to the CIRCE element. A biotinylated double-stranded DNA template containing CIRCE was attached to streptavidin-conjugated magnetic beads and used to purify CIRCE-binding complexes from a chlamydial lysate. Proteins that bound to the CIRCE affinity beads were eluted with a high-salt wash and identified in an initial screen by mass spectrometry (Table 2). Among the proteins identified were HrcA, which provided validation for this method, and the heat shock proteins, GroEL, GroES, and DnaK. Also identified were the chlamydial major outer membrane protein (MOMP), the translation factor EF-Tu, and an acyl carrier protein (AcpP).

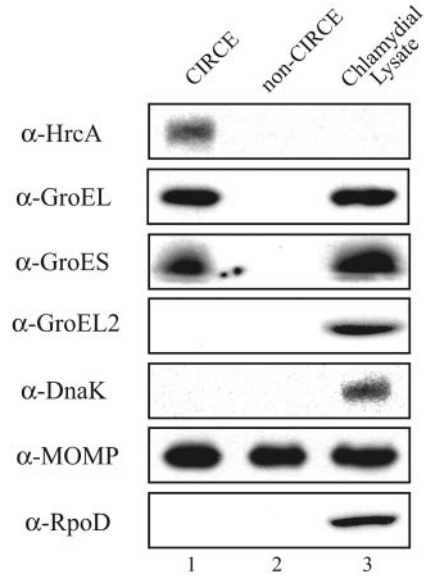

FIG. 1. Western blot analysis of DNA affinity-purified proteins. Equal amounts of lysates purified by CIRCE DNA beads (lane 1) and non-CIRCE DNA beads (lane 2) were resolved by SDS-PAGE. A diluted, crude 28-h-postinfection *C. trachomatis* serovar L2 lysate was loaded as an antibody control (lane 3). The SDS-PAGE gel was transferred to nitrocellulose and probed with the indicated antibody. Following detection, the same blot was stripped and reprobed with additional antibodies as indicated. Antibody information is provided in Materials and Methods.

We repeated this affinity purification experiment to determine if binding was specific and if the proteins were present in quantities large enough to detect by Western blotting with specific antibodies. We compared binding to CIRCE beads with binding to beads coated with non-CIRCE DNA. As shown in Fig. 1, HrcA, GroEL, and GroES were each readily detected with the CIRCE beads but not with the non-CIRCE beads, indicating specificity of binding to the CIRCE sequence. As we have not been able to detect HrcA in the original chlamydial lysate by using Western blotting with the same α-HrcA antibodies, it appears that the CIRCE affinity beads greatly enriched for HrcA. In contrast, DnaK was not detected with either set of beads, indicating that only small amounts of this heat shock protein, below the threshold detection level of Western blotting, were present in our original isolation. On the other hand, MOMP was detected with both CIRCE and non-CIRCE beads, suggesting that it bound nonspecifically. We did not examine the binding of EF-Tu and AcpP, as antibodies to these non-heat shock chlamydial proteins were unavailable. A negative control protein, RpoD, which is the major sigma factor in *Chlamydia*, did not bind to either CIRCE or non-CIRCE beads. These results indicate that chlamydial HrcA, GroEL, and GroES bound specifically to the CIRCE element, although from these experiments alone, we could not tell whether GroEL and GroES bound directly to CIRCE or indirectly through HrcA.

HrcA and GroE are part of a higher order complex in vivo. The finding that other proteins were isolated together with HrcA prompted us to compare the CIRCE binding complexes formed by the native HrcA preparation and recombinant

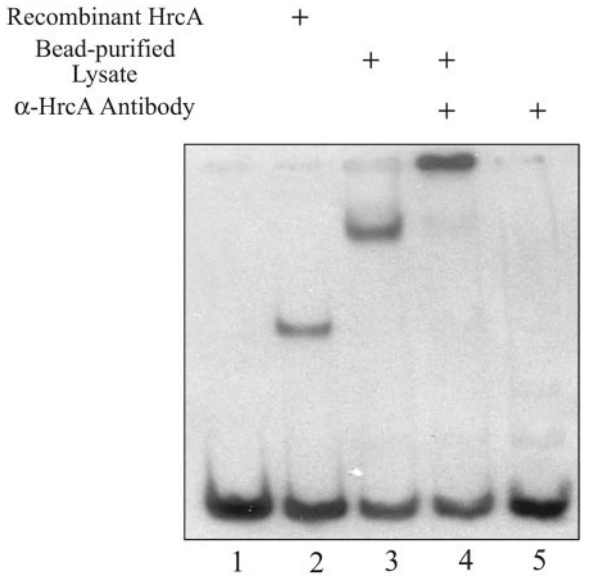

FIG. 2. Native HrcA EMSA. ^{32}P-labeled DNA probe containing the CIRCE element was incubated with several protein preparations, and the resulting complexes were resolved by native polyacrylamide gel electrophoresis. Lane 1, no protein; lane 2, recombinant HrcA; lane 3, CIRCE DNA bead-purified lysate; lane 4, CIRCE DNA bead-purified lysate with α-HrcA antibody; lane 5, α-HrcA antibody alone.

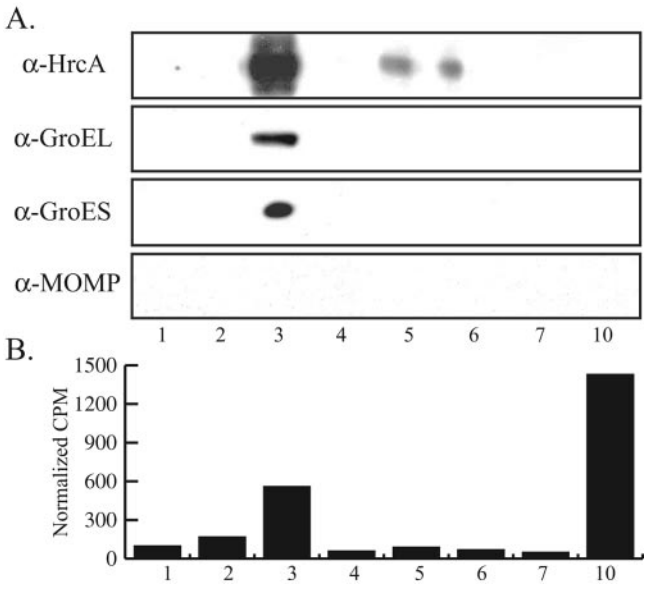

FIG. 3. EMSA protein complex analysis. A. Western blot analysis of proteins extracted from EMSA gel. Vertical lanes were cut from an EMSA gel and sectioned horizontally into 10 samples. Proteins were extracted from each gel slice sample and subjected to SDS-PAGE followed by Western blotting. Each vertical lane in the blot represents a section of the original EMSA gel lane. The blot was probed with a series of antibodies as indicated for each image. B. Quantification of ^{32}P-labeled DNA in gel samples. Gel slices generated in parallel with extracted samples described above were analyzed by liquid scintillation counting. Normalized values for ^{32}P radioactivity signal of samples corresponding to lanes in the Western blot are represented in a bar graph.

HrcA. EMSA experiments with a CIRCE DNA fragment showed that the endogenous HrcA fraction produced a gel shift of slower mobility than recombinant HrcA (Fig. 2, compare lanes 3 and 2). Some labeled probe was also retained in the well for the endogenous HrcA fraction. Heavy overexposure of the gel also demonstrated a second very faint shifted band that migrated just below the band produced by recombinant HrcA (data not shown). Taking into account that recombinant HrcA is slightly larger than the native HrcA because of the six-His tag, this faint band may represent the binding of a small amount of endogenous HrcA to CIRCE in the absence of GroEL and GroES. The addition of α-HrcA antibodies caused the complete loss of the slower mobility band and retention of the probe in the well consistent with a supershift (Fig. 2, lane 4). A control experiment with antibody alone produced no shift (Fig. 2, lane 5). These results demonstrate that the slower mobility band produced by the endogenous HrcA fraction contained HrcA but also suggested that additional proteins might account for the decreased mobility compared to recombinant HrcA.

We analyzed the EMSA products further to determine if the slower mobility band contained additional proteins. The EMSA reaction product with the endogenous HrcA fraction was resolved on a native polyacrylamide gel and divided into 10 slices according to different gel mobility. Proteins were extracted from each gel slice and identified by Western blotting with specific antibodies. In parallel, a second set of gel slices were analyzed by liquid scintillation counting to track slices with high levels of ^{32}P-labeled probe DNA. As shown in Fig. 3, HrcA was strongly detected in slice 3, which colocalized with the slower mobility band. This slice also contained GroEL and GroES but not DnaK or MOMP. Only HrcA, albeit at low levels, was detected in slices 5 and 6, confirming that the very faint second shifted band contained HrcA alone. The high levels of radioactivity detected in slice 10 were from unbound labeled DNA probe. These results demonstrate that the majority of the endogenous HrcA bound to the CIRCE operator as a complex that also contained GroEL and GroES. Only a small portion of endogenous HrcA appeared to bind to CIRCE alone.

GroEL increases binding of recombinant HrcA to CIRCE. To examine the biologic significance of this physical interaction between GroEL and HrcA, we investigated the effect of GroEL on the known functions of HrcA. We were mindful that GroEL has been reported to modulate HrcA activity in several bacterial species (22, 23, 43).

We first examined the effect of recombinant GroEL on HrcA-CIRCE binding. Figure 4A shows that in an EMSA experiment, GroEL increased the amount of probe to which HrcA bound, although we did not observe the slower mobility complex produced by the endogenous HrcA fraction. GroEL by itself did not produce a gel shift, indicating that GroEL by itself does not bind CIRCE under the conditions tested. GroEL increased HrcA-CIRCE binding over a range of HrcA concentrations (Fig. 4B). The effect was greatest at lower HrcA concentrations, with which there was as much as a fourfold increase in HrcA-CIRCE binding. Even small amounts of GroEL caused HrcA to produce a complete shift, leaving no free probe, which is in contrast to our previous finding that HrcA alone was not able to completely bind to the CIRCE probe even at high HrcA concentrations. These results indicate

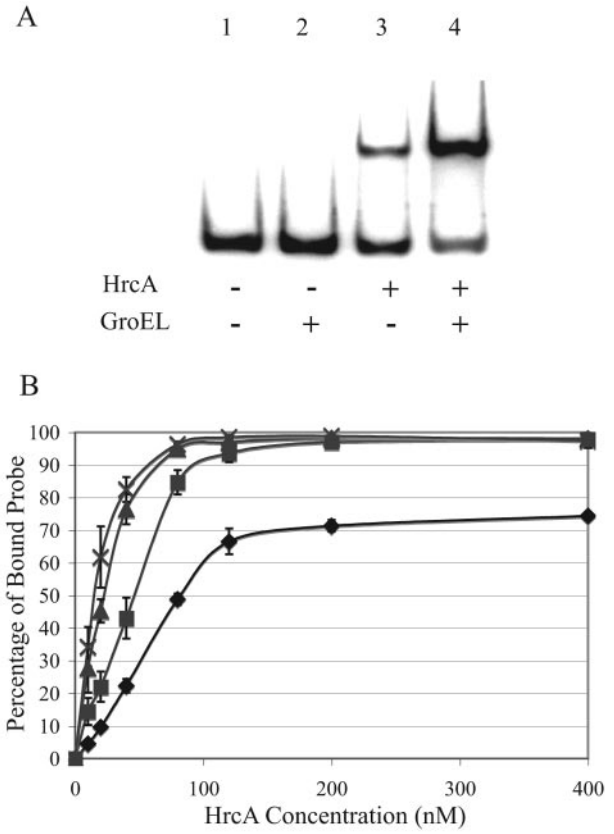

FIG. 4. Effect of GroEL on recombinant HrcA DNA binding activity. A. ^{32}P-labeled DNA probe containing the CIRCE element was incubated with several protein preparations, and the resulting complexes were resolved by native polyacrylamide gel electrophoresis. Lane 1, no protein; lane 2, 40 nM GroEL; lane 3, 75 nM HrcA; lane 4, 75 nM HrcA and 40 nM GroEL. B. Graph showing the effects of several concentrations of GroEL on HrcA DNA binding activity. HrcA concentrations are indicated on the *x* axis. GroEL concentrations are as follows: no GroEL (◆), 40 nM GroEL (■), 75 nM GroEL (▲), 120 nM GroEL (×). Data were combined from three independent reactions, and error bars are standard deviations from the means.

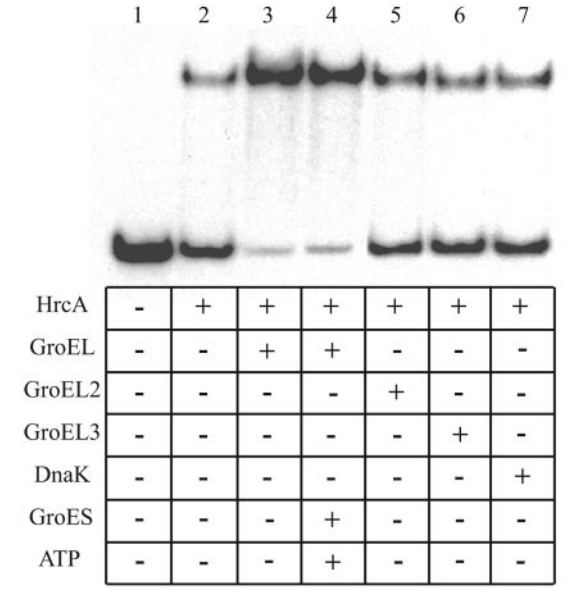

FIG. 5. Effect of chlamydial heat shock proteins on recombinant HrcA. ^{32}P-labeled DNA probe containing the CIRCE element was incubated with several protein preparations, and the resulting complexes were resolved by native polyacrylamide gel electrophoresis. Lane 1, no protein; lane 2, 70 nM HrcA; lane 3, 70 nM HrcA and 50 nM GroEL; lane 4, 70 nM HrcA and 50 nM GroEL, 50 nM GroES, and 100 μM ATP; lane 5, 70 nM HrcA and 100 nM GroEL2; lane 6, 70 nM HrcA and 100 nM GroEL3; lane 7, 70 nM HrcA and 100 nM DnaK.

that GroEL increases HrcA-CIRCE binding and may be necessary for complete transcriptional repression of the heat shock genes by HrcA.

We also tested whether other heat shock proteins, including the two additional chlamydial GroEL paralogs (16, 39), could modulate HrcA-CIRCE binding. GroEL2, GroEL3, GroES, and DnaK did not bind to the CIRCE probe (data not shown). Several concentrations of each protein were titrated with HrcA (data not shown), and the protein concentration that showed the maximum effect on HrcA activity is shown in Fig. 5. The addition of GroES and/or ATP to GroEL did not augment the effect of GroEL on HrcA (Fig. 4B and 5, compare lanes 3 and 4). Unlike GroEL, neither GroEL2 nor GroEL3 increased binding of HrcA to the CIRCE probe. The addition of GroES did not affect the activity of GroEL2 or GroEL3 (data not shown). DnaK had no effect on HrcA binding, arguing against a role for DnaK in regulating HrcA activity in *Chlamydia*, in contrast to reports of the situations for other bacteria (17, 33).

To address the possibility that our recombinant GroEL and GroES might not form an active chaperonin, we also purified intact chlamydial GroEL and GroES from *E. coli* under conditions shown to produce a functional GroE chaperonin complex (25, 30). This copurified GroESL complex increased HrcA binding to CIRCE by a similar amount as with GroEL alone but did not produce the slower mobility complex seen with the endogenous HrcA complex (data not shown).

These results show that of the major chlamydial heat shock proteins, only GroEL (but not GroES), the two GroEL paralogs, and DnaK can modulate the binding of HrcA to CIRCE. Augmentation of HrcA-CIRCE binding by GroEL did not appear to require hydrolysis of ATP and was unaffected by supplementation with additional ATP.

The HrcA/GroE complex could not be reassembled in vitro after disruption of the native complex. We performed further experiments on the native HrcA/GroE complex to learn more about the different CIRCE binding complexes obtained with native and recombinant proteins. As we were not able to assemble the HrcA/GroE complex in vitro from recombinant proteins, we were interested in seeing if the native complex could be disassociated and reassembled. We disrupted the CIRCE-bead-purified, native HrcA/GroE complex with 6 M urea, removed the urea, and then performed an EMSA experiment to examine the complex formed with CIRCE. With the disruption of the native HrcA/GroE complex, the gel shift previously shown to represent the HrcA/GroE/CIRCE supercomplex disappeared and was replaced by a band that migrated just a little faster than the gel shift produced by recombinant HrcA (Fig. 6, compare lanes 2 through 4). This slight difference in migration is consistent with the extra 3-kDa histidine tag on the recombinant protein. The addition of recombinant

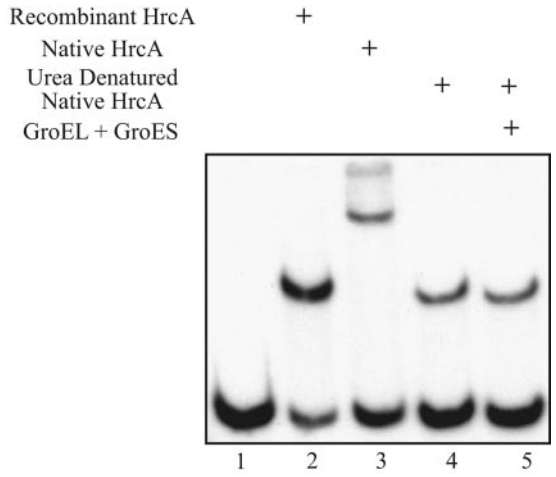

FIG. 6. DNA binding activity of native and denatured HrcA. ^{32}P-labeled DNA probe containing the CIRCE element was incubated with several protein preparations and the resulting complexes were resolved by native polyacrylamide gel electrophoresis. Lane 1, no protein; lane 2, recombinant HrcA; lane 3, CIRCE DNA bead-purified lysate; lane 4, CIRCE DNA bead-purified lysate denatured in 6 M urea followed by dialysis against storage buffer; lane 5, CIRCE DNA bead-purified lysate denatured in 6 M urea followed by dialysis against storage buffer with the addition of 50 nM GroEL and 50 nM GroES.

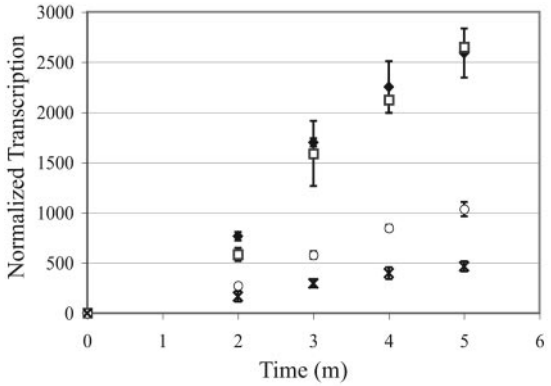

FIG. 7. Effect of GroEL on recombinant HrcA-mediated transcriptional repression. The graph represents the results of in vitro transcription of CIRCE-regulated *dnaK* promoter in the presence of HrcA and GroEL. Transcription was performed using partially purified chlamydial RNA polymerase under DNA substrate-limiting conditions in the presence of ^{32}P-labeled nucleotides. RNA products are resolved by urea-PAGE. Data from three independent reactions were combined for each time point, and error bars are standard deviations from the means. Recombinant proteins added to reactions are as follows: no recombinant protein (◆), 400 nM GroEL alone (□), 800 nM HrcA alone (○), and 800 nM HrcA and 400 nM GroEL (×).

chlamydial GroEL and GroES to the renatured HrcA did not restore the slower mobility band or significantly alter the binding of HrcA to CIRCE (Fig. 6, lane 5). These results show that native HrcA was able to bind to CIRCE in a manner similar to that of recombinant HrcA. However, the HrcA/GroE complex was not readily reassembled in vitro from the native components.

GroEL increases transcriptional repression by recombinant HrcA. As recombinant GroEL was able to increase the binding of HrcA to CIRCE, we also tested if GroEL could enhance HrcA-mediated repression, an effect not previously studied in vitro for any bacterial system. In vitro transcription reactions were performed under DNA substrate-limiting conditions, and RNA samples were collected for analysis over a time course of up to 15 min. Data points in the linear range are plotted in Fig. 7. HrcA-mediated repression was enhanced from a 2.7-fold decrease in the rate of transcription to a 6.9-fold decrease when GroEL was added. GroEL by itself did not have a significant effect on the rate of transcription. These results demonstrate that the effect of GroEL in increasing the binding of HrcA to CIRCE can also be measured as an increase in HrcA-mediated transcriptional repression.

DISCUSSION

In this report, we provide evidence that the chlamydial *dnaK* and *groE* operons are regulated by the GroE chaperonin through direct interactions with the HrcA repressor. DNA affinity purification using the CIRCE operator allowed us to greatly enrich for HrcA and to identify several other copurifying chlamydial proteins, demonstrating the utility of this method for isolating DNA binding proteins and their associated factors. Of the proteins that were isolated together with HrcA, only GroEL and GroES could be demonstrated to form a complex with HrcA that bound DNA. This is the first reported isolation of an active higher-order complex containing native HrcA, GroEL, and GroES from any bacterium. Prior to this work, the strongest support for an interaction between HrcA and GroEL has been the ability to pull down GroEL with a recombinant *Bacillus subtilis* HrcA protein that had been engineered with several mutations for improved solubility (31).

The interaction between HrcA and GroEL appears to be biologically significant, as GroEL enhanced both the binding of HrcA to CIRCE and the level of HrcA-mediated transcriptional repression. In fact, our results strongly suggest that GroEL is necessary for the complete repression of heat shock promoters, since HrcA was unable to bind to all the labeled CIRCE DNA unless GroEL was present. We found that the majority of native HrcA purified by binding to CIRCE was in a complex with GroEL and GroES and that only a small proportion was able to bind to CIRCE in the absence of the other heat shock proteins. Curiously, we were unable to reconstitute in vitro a complex containing HrcA, GroEL, and GroES from either the recombinant proteins or the native constituents after disruption of the complex. Our finding that GroEL enhanced HrcA-CIRCE binding may provide some evidence for the formation of the ternary complex; it is possible that the in vitro assembled complex was unstable in the EMSA assay and that only a more-stable HrcA-CIRCE product was detected. The stability of EMSA products, especially ternary protein complexes, is known to vary depending on the conditions used in the EMSA binding reactions and during gel electrophoresis (32, 34, 45). Our findings suggest that there is something different about how the native HrcA complex was assembled, and we hypothesize that different conditions or additional factors may be involved. Of the three GroEL paralogs in *Chlamydia*, only the GroEL protein encoded by CT110 was able to physically interact with HrcA and modulate HrcA function. The

two other GroEL paralogs present in all chlamydial species do not contain CIRCE elements upstream of their genes (A. C. Wilson and M. Tan, unpublished observations) and are not accompanied by an adjacent *groES* gene (39). Other studies have also suggested that GroEL2 and GroEL3 are unlikely to function as chaperonins, as they are poorly conserved with bacterial GroEL proteins and do not functionally interact with GroES (16). Although we detected DnaK in the initial mass spectrometry screen of proteins purified by binding to CIRCE-coated beads, this heat shock protein was present in amounts too small to confirm by Western blotting. Furthermore, recombinant DnaK was unable to modulate HrcA function. These observations are consistent with reports that in *B. subtilis*, DnaK plays no role in regulation of HrcA (23).

Our results support a general model for the regulation of HrcA-mediated repression by GroE levels that was first put forward by Mogk et al. (23). In the so-called GroE titration model, GroE was proposed to stabilize HrcA-CIRCE interactions, with the corollary that HrcA-mediated transcriptional repression would be sensitive to GroE levels. Thus, conditions such as stress, which titrate away GroE to correct protein misfolding in its canonical function as a molecular chaperone, are predicted to cause derepression of heat shock gene expression. In vivo GroE levels have been shown to modulate the degree of repression by HrcA in other bacteria (43, 48, 49). Our results, however, are the first to demonstrate that GroEL together with GroES can form a stable physical interaction with the HrcA-CIRCE complex and that GroEL can enhance transcriptional repression by HrcA in vitro.

The role of GroEL in regulating HrcA-mediated repression provides a mechanism that allows *Chlamydia* to sense cellular stress and respond with a transient, self-limiting increase in GroEL expression. GroEL, in effect, is able to homeostatically regulate its own expression. Although elevated temperature has been shown to induce the heat shock response in *Chlamydia* (15) along with up-regulation of *groE* transcription (9, 42), there are reasons to believe that it is not the main source of cellular stress. An increased temperature sufficient to induce the heat shock response in *Chlamydia* (9) could not relieve HrcA-mediated transcriptional repression in vitro (51). It is also worth noting that chlamydiae reside inside the relatively thermostable environment of a mammalian cell throughout the replicative cycle. Other stress conditions, such as ethanol, and puromycin treatment, have been shown to titrate away GroE in *B. subtilis* and relieve transcriptional repression by HrcA in vivo (24).

The forms of cellular stress that may be more relevant in *Chlamydia* include nutrient deprivation and cytokine exposure, which have each been shown to induce chlamydiae to enter a persistent infectious state (14). Although GroEL expression is increased during persistence, the up-regulation is sustained rather than transitory, with high levels of *groE* mRNA and GroEL protein (3, 4, 7, 12). The means by which the normal GroEL feedback loop is overcome may provide valuable insight into the mechanism of chlamydial persistence.

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