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Characterizing the polymeric status of *Helicobacter pylori* heat shock protein 60

Ching-Yi Lin [a], Yu-Shan Huang [a], Chi-Han Li [a], Yuan-Ting Hsieh [a], Nu-Man Tsai [c], Pei-Juin He [a], Wei-Tung Hsu [a], Yi-Chen Yeh [a], Fang-Hsing Chiang [a], Ming-Shiang Wu [b], Chia-Ching Chang [a], Kuang-Wen Liao [a,*]

[a] Department of Biological Science and Technology, National Chiao-Tung University, Hsin-Chu, Taiwan
[b] Department of Internal Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan
[c] School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan

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ABSTRACT

Helicobacter pylori heat shock protein 60 (HpHsp60) was first identified as an adhesion molecule associated with *H. pylori* infection. Here we have analyzed the structure of HpHsp60 via amino acid BLAST, circular dichroism, and electrophoresis and the results indicate that most recombinant HpHsp60 molecules exist as dimers or tetramers, which is quite different from *Escherichia coli* Hsp60. Treatment of human monocytic cells THP-1 with HpHsp60 was found to up-regulate a panel of cytokines including IL-1α, IL-8, IL-10, IFN-γ, TNF-α, TGF-β, GRO, and RANTES. Carboxymethylated HpHsp60 molecules with a switched oligomeric status were able to further enhance NF-κB-mediated IL-8 and TNF-α secretion in THP-1 cells compared to unmodified HpHsp60 molecules. These results indicated that the oligomeric status of HpHsp60s might have an important role in regulating host inflammation and thus help facilitate *H. pylori* persistent infection.

Introduction

Helicobacter pylori (*H. pylori*) has been shown to play a pathogenic role in gastric disease. This organism is associated with the pathogenesis that occurs in certain upper gastrointestinal disorders including chronic gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1]. Various virulent factors, such as CagA [2], Vac A [3], BabA [4], and *rocF*-gene encoded arginase [5], have been demonstrated to play significant roles in *H. pylori* colonization and persistent infection.

The expression of heat shock protein (Hsp) 60 (also known as chaperonin (Cpn) 60) by *H. pylori* has been shown to have a role as an adhesion molecule that interacts with host gastric epithelial cells and mucin [6]. This results would seem to be contradicted by the fact that Hsp60 proteins have been found to show a very high level of sequence conservation across many species and by the fact that this group of molecules shows extreme immunogenicity [7]. As a potentially important *H. pylori* antigen, Hsp60 might activate myeloid cells to produce a range of pro-inflammatory cytokines including IL-1β [8], IL-6 [8,9], and IL-8 [8,10], and it might also stimulates KATO III human gastric epithelial cells to secrete IL-8 [11].

Although the structure of *H. pylori* Hsp60 itself has not been investigated in depth, previous work has established the structure of *Escherichia coli* Hsp60 [12]. *E. coli* Hsp60 is a porous cylinder of 14 subunits made up of two approximately 7-mer rotationally symmetrical rings stacked back-to-back. Due to the profound functional stability of the bacterial chaperonin system and their evolutionary conservation among species, both mammalian and prokaryotic Hsp60s have been assumed to function in a similar way. However, an increasing number of studies are showing that there are variations in terms of structure and function between the Hsps of various species [13–15]. These differences possibly reflect evolutionary adaptation of a specific homolog to a particular microenvironment and to various endogenous substrates.

In this study, we cloned and expressed recombinant *H. pylori* Hsp60 (rHpHsp60) and analyzed the proteins molecular structure using sequence alignment, gel electrophoresis and circular dichroism. Although the sequence of Hsp60 contains many highly conserved amino acid sequences across many species and HpHsp60 has similar a secondary structure composition to other Hsps, the oligomerization of the Hsp60s from *H. pylori* and *E. coli* are quite different. This difference seems to be reflected in HpHsp60's effect on the pro-inflammatory cytokine profile produced by the monocytic cell line THP-1 after treatment with HpHsp60. Blocking the thiol group of a Cys residue within

* Corresponding author. Address: Department of Biological Science, National Chiao-Tung University, Room 205 Zhu-Ming Building, 75 Bo-Ai Street, Hsin-Chu, Taiwan. Fax: +886 3 5729288.
E-mail address: kitchhen@yahoo.com.tw (K.-W. Liao).

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HpHsp60 altered its molecular conformation and its ability in terms of cytokine induction and NF-κB activation. This would seem to suggest that HpHsp60 might have a role in regulating the gastric inflammatory responses via changes in the protein's oligomer composition.

Materials and methods

Cell culture. The human monocytic cell line THP-1 was obtained from Bioresource Collection and Research Center (Hsin-Chu, Taiwan) and maintained in RPMI media supplemented with 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 μg/ml of penicillin/streptomycin, and 10% heat-inactivated FBS (Biological Industries, Haemek, Israel) at 37 °C, 5% CO_2.

Amino acid sequence analysis. The amino acid sequences of *H. pylori* and *E. coli* Hsp60 were aligned using the Vector NTI Suite 9 software (Invitrogen, USA). Variable amino acid positions and their relative frequencies were identified from the alignment.

Expression and purification of H. pylori Hsp60. A DNA fragment containing the Hsp60 gene was amplified from *H. pylori* genomic DNA (the clinically isolated strain was kindly provided by Dr. Ming-Shiang Wu, Department of Internal Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan) by polymerase chain reaction and cloned into the T7 promoter-driven pET 30a (+) expression vector (Novagen, Darmstadt, Germany). The sequences of HpHsp60 was verified by sequencing and submitted to GenBank (Accession No. DQ674277). The protein were then expressed in *Escherichia coli* (BL21 strain) and purified using

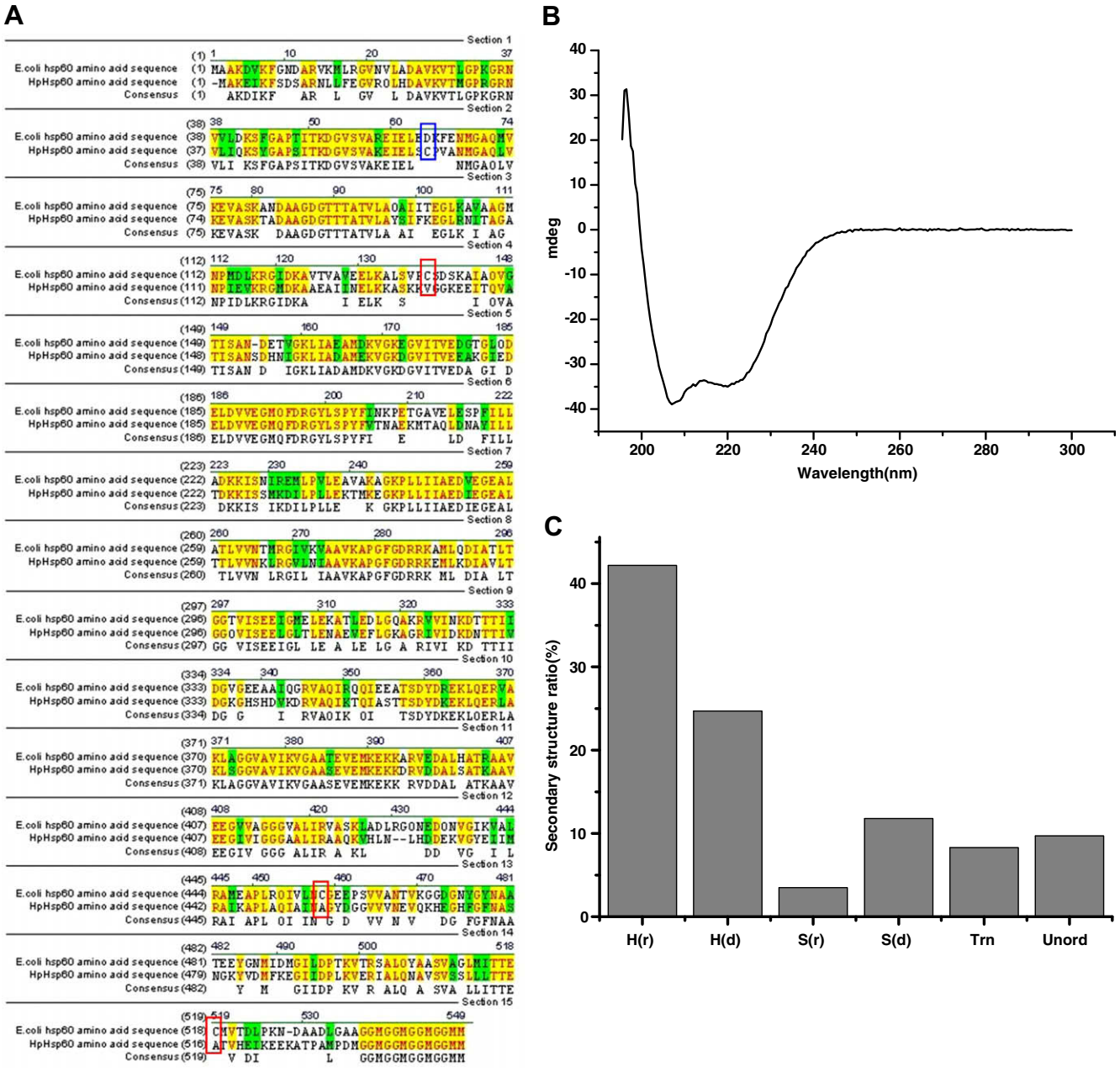


Fig. 1. Primary and secondary structural analysis of *H. pylori* Hsp60. (A) Sequence alignment of *H. pylori* and *E. coli* Hsp60. The amino acid sequences of *E .coli* and *H. pylori* Hsp60 were retrieved from Pubmed database and analyzed by Vector NTI Suite 9 software. The blue and red frames indicated the cysteines within *H. pylori* and *E. coli* Hsp60. CD spectra (B) and secondary structure estimation (C) of *H. pylori* Hsp60. Measurements were performed in 0.1 M phosphate buffered saline (PBS) at pH 7.4. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this paper.)

HisTrap affinity (Ni–NTA) chromatography (General Electric, NY, USA) followed by Sephadex G-25 column (General Electric, NY, USA) to remove any salts associated with proteins. The purity of the HpHsp60 was determined by SDS–PAGE and Western blot assay (detected by HRP conjugated goat polyclonal antibody to polyhistidine; Novus, CO, USA) then the protein's identity was confirmed by mass spectrometry (MALDI-TOF-MS).

Carboxymethylation of H. pylori Hsp60. Carboxymethylation was carried out by dissolving 5 mg of *H. pylori* Hsp60 in 5 ml of 0.1 M Tris–HCl buffer (pH 8.6) containing 5.4 M urea, and 1% v/v β-mercaptoethanol. After the solution was flushed with nitrogen and then incubated at room temperature for 2 h, 20 mg of iodoacetic acid was added and the reaction mixture maintained at a pH of 8.6 by the addition of 0.1 M NaOH; incubation continued for another 2 h. Finally, the carboxymethylated (CM)-HpHsp60 was dialyzed against 1× PBS buffer (pH 7.4) in order to desalt the solution.

Gel electrophoresis. Reducing SDS–PAGE was carried out using 10% 1 mm thick slab gels with the Bio-Rad MiniProtean™ 3 system (Bio-Rad, CA, USA). The final volume of the resolving gel was 4.0 ml with 1.3 ml of 30% acrylamide solution, 1 ml of 1.5 M Tris–HCl (pH 8.8), 1.6 ml of distilled water, 40 μl of 10% SDS, 20 μl of 10% APS, and 3.6 μl of TEMED. Proteins mixed with 3× sample buffer (30% v/v glycerol, 37.5% v/v of 0.5 M Tris–HCl (pH6.8), 15% v/v β-mercaptoethanol (2-Me), 0.66% w/v, and 0.000075% w/v bromophenol blue) were incubated in 95 °C for 15 min and then loaded into wells and subjected to electrophoresis. Procedures similar to the above were followed when carrying out non-reducing SDS–PAGE except that β-mercaptoethanol was not present in the 3× sample buffer. Native-PAGE was performed as a sequential process on 7.5% native gels in which SDS was absent from 1× sample tracking dye, stacking/separating gels, and electrophoresis running buffer. Proteins analyzed by native gel were directly incubated with 1× sample tracking dye without boiling.

Circular dichrosim (CD) spectroscopy. CD measurements of *H. pylori* Hsp60 were conducted on an AVIV Model410 Circular Dichroism spectropolarimeter. Instrument optics and sample chamber were continuously flushed with 10 l/ml of dry N_2 gas. The purified proteins were dissolved in 0.1 M phosphate buffered saline (PBS) at pH 7.4 to obtain a protein concentration of 0.5 mg/ml. Spectra were recorded in the far-ultraviolet (UV) region (200–260 nm). Protein samples were measured at room temperature in a 1 mm pathlength quartz cell (300 μl) using a scan speed of 50 nm/min, a time response of 1 s, and a bandwidth of 1 nm; analysis was carried out on an average of eight scans.

Human protein cytokine array. THP-1 cells (10^5/well) were seeded in 24-well culture plates with 1 ml growth medium per well and incubated at 37 °C in a 5% CO_2 atmosphere for 2 h. Next, 10 μg of HpHsp60 was added to the appropriate wells. After 16-h incubation, the supernatants were harvested and assayed by human protein cytokine array (RayBiotech, GA USA) according to the manufacturer's instructions. Membranes were briefly blocked with a blocking buffer, and then 1 ml of medium from either the HpHsp60 treated or untreated THP-1 cell cultures were added and incubated at room temperature for 2 h. After incubation, the membranes were treated for 1 h with biotin-conjugated anti-cytokine antibodies, reacted with horseradish peroxidase–streptoavidin and then developed using ECL.

IL-8 and TNF-α production inducted by H. pylori Hsp60 treatments of THP-1 cells. THP-1 cells were seeded under the same conditions described previous above. Following this, 10 μg of unmodified or CM-HpHsp60 protein was added into the appropriate wells for a 16-h incubation period. Supernatants were then harvested and the supernatant assayed to assess the level of TNF-α and IL-8; this was done using the DuoSet® ELISA development system and carried out according to the manufacturer's specifications (R&D Systems, MN, USA).

NF-κB-dependent reporter gene expression assay. A plasmid containing a minimal human NF-κB promoter driving the hrGFP gene (pNF-κB-hrGFP) (Strategene, USA) was used to investigate NF-κB activity in *H. pylori* Hsp60-treated THP-1 cells. Transfections of THP-1 cells with pNF-κB-hrGFP were carried out by the electroporation method using a Multiporator® (Eppendorf, Hamburg, Germany). THP-1 cells (3×10^6 cells/ml) were incubated with 75 μg of plasmid followed by electroporation (350 V, 80 μs, 1 pulse). The cells were harvested at 48 h after electro-transfection and hrGFP expression was assayed by flow cytometer.

Statistical analysis. The results are presented as means ± SEM. Significant differences were evaluated with by the Student's *t*-test. A p value of <0.05 was considered statistically significant.

Results

Analysis of amino acid sequences of E. coli and H. pylori Hsp60

The sequences of *E. coli* and *H. pylori* hsp60 proteins were retrieved from the NCBI Protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein). The accession numbers of *E. coli* and *H. pylori* Hsp60 proteins are AAS75782 and DQ674277, respectively. The protein sequence alignment showed that the similarity and identity between HpHsp60 and *E. coli* Hsp60 are 73.0% and 59.9%, respectively (Fig. 1A). However, there is only one cysteine in the protein sequence of HpHsp60 (blue frame) while there are

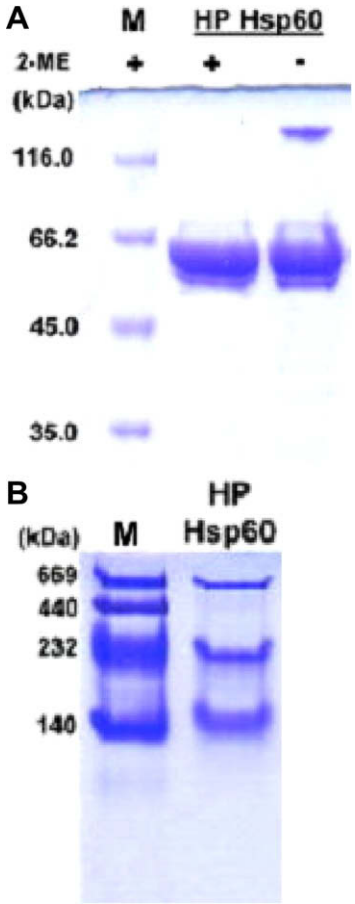


Fig. 2. Electrophoresis profile of HpHsp60. (A) Reducing and non-reducing (with and without 2-Me) SDS–PAGE. (B) Native-PAGE. M, markers.

three cysteine residues present within the *E. coli* Hsp60 sequence (red frame).

The results of the circular dichroism measurements provided an overview of the secondary structures of the *H. pylori* Hsp60 protein. Spectra were recorded in 0.1 M phosphate-buffered saline (PBS) at pH 7.4 with a protein concentration of 0.5 mg/ml (Fig. 1B). The quantitative evaluation of the data in terms of secondary structure components is shown in Fig. 2B. The CD spectra of *H. pylori* Hsp60 displayed a major α-helix conformation composed of 42.2% regular and 24.7% distorted forms, 15.3% β-sheet (with 3.5% regular and 11.8 distorted forms), 8.3% turns and 9.7% unordered structure (Fig. 1C).

Analysis of the effect of the disulfide bonding on H. pylori Hsp60 structure

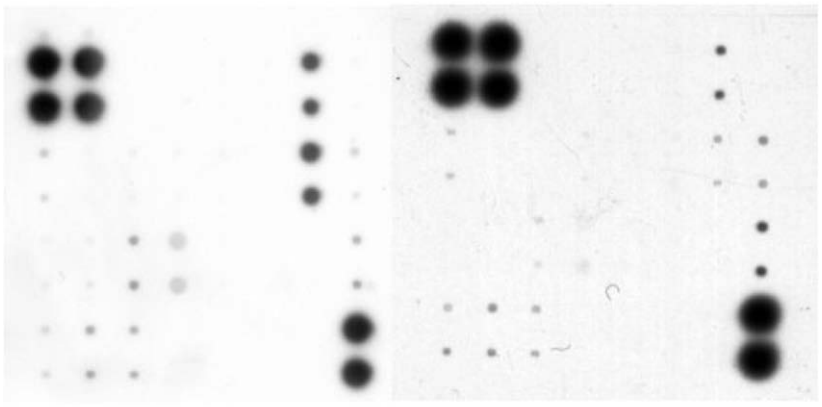
The *H. pylori* Hsp60 recombinant proteins (rHpHsp60) were analyzed by PAGE with or without 2-ME treatments to reveal their potential structure. As seen in Fig. 2A, most rHpHsp60 proteins consisted of monomers and only a few dimers were formed by intermolecular disulfide bonds. The results of the native PAGE indicated that there were three major forms of rHpHsp60. Based on the molecular weight markers, recombinant *H. pylori* Hsp60 is able to undergo multiple oligomerization to give dimers, tetramers, and decamers (Fig. 2B). The results indicated that the cysteine residue does not seem to be involved in the oligomerization of rHpHsp60 via intermolecular disulfide bonds.

Cytokine expression profiles of THP-1 cells induced by HpHsp60

The microarray membranes were scanned after reacted with the culture medium of THP-1 cells with or without HpHsp60 treatment (Fig. 3A) and the density of each spot was assessed by densitometry. The relative intensities of the cytokine profiles were normalized appropriately against control spots on each membrane.

	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
3	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
4	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
6	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
7	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos
8	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

A



HpHsp60 treated **NC**

B **Human inflammatory cytokine levels elevated from HpHsp60-treated THP-1 cells**

	Human Cytokine Protein	Fold Increase
IL-8	Interleukin-8	11.67 fold
GRO	Growth-related oncogene	2.24 fold
IL-10	Interleukin-10	2.03 fold
TNF-α	Tumor necrosis factor-α	1.86 fold
IL-1α	Interleukin-1α	1.86 fold
IFN-γ	Interferon-γ	1.79 fold
RANTES	Regulated on activation normal T cell expressed and secreted	1.52 fold
TGF-β1	Transforming growth factor-β1	1.5 fold

Fig. 3. Human cytokines are up-regulated when THP-1 monocytic cells are treated with HpHsp60. Human protein-array analysis was used to determine changes in cytokine release from HpHsp60-stimulated THP-1 cells. (A) Data from the array analyzed using filter 1 in which the cells were treated with HpHsp60 (Left) and using filter 2, the medium control (Right). (B) The intensities of the relative expression levels of cytokines were quantified by densitometer (GE, USA). The values from scans were normalized based on the intensity of control spots on the filter corners, and the increases in specific cytokines were shown.

The data showed that the conditioned medium from HpHsp60-treated THP-1 cells displayed up-regulation of several different target cytokines including IL-1α (1.86-fold comparing to NC), IL-8 (11.67-fold), IL-10 (2.03-fold), TNF-α (1.86-fold), IFN-γ (1.79-fold), and TGF-β1 (1.5-fold). Furthermore, the chemokines such as GRO (2.24-fold) and RANTES (1.52-fold) were also elevated in response to HpHsp60 (Fig. 3B).

The effect of the cysteine residue in H. pylori Hsp60 on protein structure and the induction of pro-inflammatory cytokines

Although the cysteine residue contained within *H. pylori* Hsp60 does not seem to be involved in the formation of intermolecular disulfide bond, the differences between *E. coli* and *H. pylori* Hsp60 in terms of cysteine residues might be relevant to how the protein functions in gastric disease. Therefore, the thiol group of the single cysteine residue within the *H. pylori* Hsp60 was carboxymethylated and analyzed to determined oligomerization. The migration rate of CM-modified HpHsp60 became slower than that of unmodified rHpHsp60 and the status of oligomeric structure changed when the only thiol group in HpHsp60 was switched to the methyl group (Fig. 4A).

To determine whether the conformation change induced by carboxymethylation of Cys was able to influence on the activity of HpHsp60, the pro-inflammatory cytokine releases of THP-1 cells induced by unmodified and CM-modified HpHsp60 were compared. The results showed CM-modified HpHsp60 elicited greater pro-inflammatory cytokine secretion, with IL-8 and TNF-α production being significantly elevated (Fig. 4B and C, $p < 0.05$) compared to unmodified rHpHsp60. It is known that activation of the NF-κB pathway results in the transactivation of multiple responsive genes that contribute to the inflammatory phenotype including IL-8 [16] and TNF-α [17] in monocytes. Therefore, the activation status of NF-κB was investigated and it was found that CM-modified HpHsp60 further increased NF-κB activation compared to unmodified rHpHsp60 (Fig. 4D, $p < 0.05$).

Discussion

Hsp60s are among the most conserved proteins in all living organisms, prokaryotic and eukaryotic. X-ray-determined structures from *E. coli* [12] have revealed that Hsp60 oligomers form a porous cylinder of 14 subunits that consists of two approximately sevenfold rotationally symmetrical rings stacked back-to-back. Each Hsp60 subunit contains three structurally distinct domains: a large equatorial domain that forms the foundation of the assembly at the waist and brings the ring together, an apical domain that forms the end of the cylinder, and a small intermediate domain that connects the two. However, although previous studies have been regularly shown that Hsp60s (otherwise named GroEL) from various prokaryotes and even from yeast [18] and pea chloroplasts [19] have a similar structure by electromicroscopy, no current evidence indicates that all Hsp60s possess exactly the same structural pattern across all the truly complicated microenvironments found in diverse species. The alignment in Fig. 1 between the Hsp60 amino acid sequences of *H. pylori* and *E. coli* shows over 70% identity. It also shows that the two Hsp60 genes have a close relationship evolutionarily, but is unable to indicate whether they have the same activity or state of oligomerization. Furthermore, it has been shown that the ATP bound to GroEL is in direct contact with

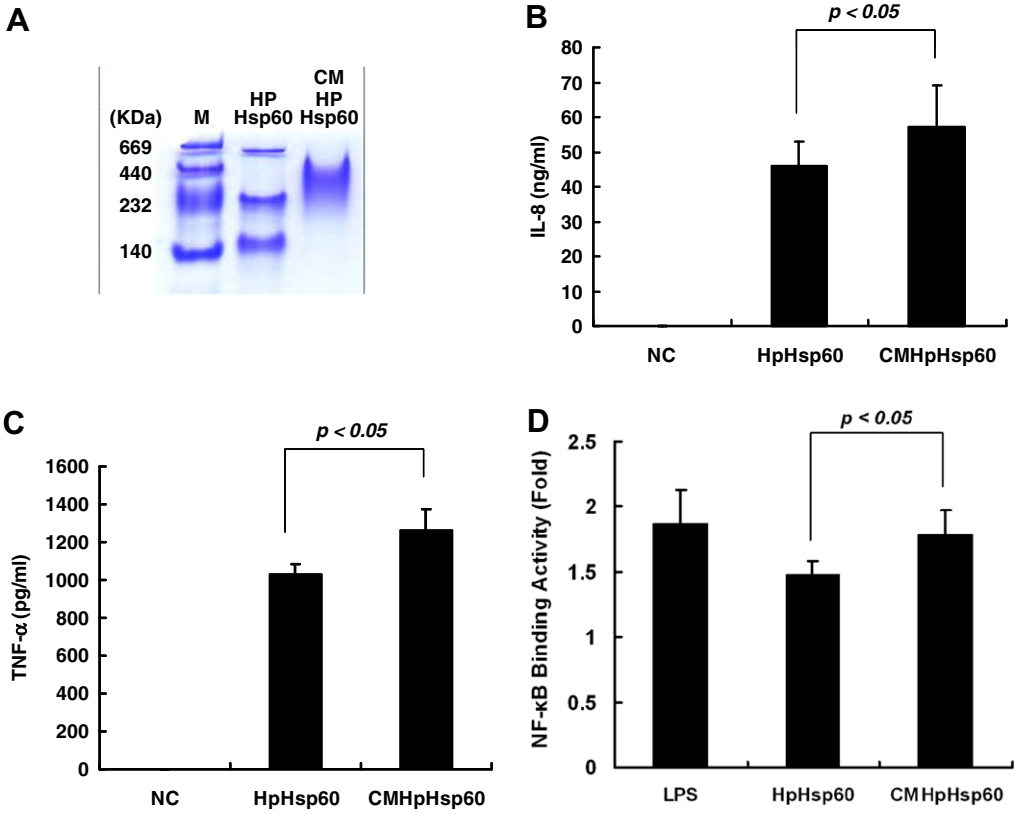


Fig. 4. The effect of rHpHsp60 versus CM-HpHsp60s on pro-inflammatory cytokine induction and NF-κB transcriptional activity. (A) Native-PAGE analysis of HpHsp60 and carboxymethylated (CM)-HpHsp60. (B,C) THP-1 cells were incubated with rHpHsp60 (10 μg/ml) for 16 h at 37 °C in 5% CO_2 and conditioned media were harvested to allow the levels of IL-8 (B) and TNF-α (C) to be determined by sandwich ELISA. Data are expressed as means ± SEM for three replicates from one of three representative experiments. (*$p < 0.05$.) (D) THP-1 cell were transiently transfected with a NF-κB-dependent reporter plasmid (pNF-κB-hrGFP) and cotreated with either oligomeric or monomeric HpHsp60s. The resulting fluorescence was analyzed by flow cytometer.

Cys-137 [20]; however, HpHsp60 did not have an equivalent cysteine residue. This suggests that even though there is high conservation in terms of protein sequence between the two species, these Hsp60s may have different activities and/or conformations. In this context, the oligomerization of *H. pylori* Hsp60 analyzed by native gel electrophoresis demonstrated that it is very different from *E. coli* Hsp60, since most HpHsp60 formed dimers and tetramers rather than the heptameric structure of *E. coli* Hsp60 (Fig. 2B).

The Hsp60 class is one of the two families of chaperonins that have been identified within the bacterial cytoplasm (GroEL) and is also present in the endosymbiotically derived mitochondria (Hsp60) and chloroplasts [21]. Being chaperonins, Hsp60s are essential to mediating ATP-dependent polypeptide chain folding. In addition to assisting with protein folding, Hsp60 proteins are also a potent immune regulator [22,23]. The conserved sequences and multifaceted properties of Hsp60s imply that this protein is important to helping all species adapt to certain abnormal circumstances. Therefore, differences in oligomerization pattern between species are reasonable given that their environments can vary greatly.

It is known that Hsp60 plays important roles in triggering host immune responses under certain circumstances. Several microbial homologs have been reported to elicit pro-inflammatory responses. The responses usually comprise the induction of inflammatory mediators such as IL-6, TNF-α and NO [22] and the activation of the T helper cells type 1 by promoting cytokines IL-12 and IL-15 [24].

The cytokine profile induced by *H. pylori* Hsp60 during monocyte stimulation is shown for the first time in this study. In a similar manner to Hsp60s derived from other species [8], *H. pylori* Hsp60 is able to stimulate THP-1 monocytes to produce IL-1α, IL-8, and TNF-α, which are involved in managing host inflammation. Furthermore, *H. pylori* Hsp60 also gives rise to IL-10 and TGF-β1 induction, which potentially can manipulate immune regulation. Finally, HpHsp60 induces GRO and RANTES secretion, which is associated with chemoattraction [25], cellular communication, survival and differentiation [26]. Although HpHsp60 shares ~70% similarity with *E. coli* Hsp60, their physiological functions seem to be different. Our findings support previous reports indicating that, although *H. pylori*, *Chlamydia* and human Hsp60s also have highly similar amino acid sequences, their potency in terms of cytokine inductions (IL-1β, IL-6, and IL-8) and vascular endothelial cell adhesion protein expression are quite different from each other [8].

Although the single cysteine residue within HpHsp60 was found not to be involved in the formation of an intermolecular disulfide bond, the residue did contribute to HpHsp60 structural integrity and is relevant to production of the pro-inflammatory cytokines IL-8 and TNF-α. *H. pylori* Hsp60 has been found to induce IL-8 release in both gastric epithelial cells [11] and monocytes/macrophages [10] through the Toll-like receptor (TLR)-2/4-mediated signal pathway. By engagement with TLR-2/TLR-4, HpHsp60 activates mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p38 and then induces the nuclear translocation of NF-κB to trigger TNF-α and IL-8 production [10]. Our results are consistent with these previous reports because we show HpHsp60 is able to significantly stimulate IL-8 and TNF-α production in THP-1 cells via NF-κB activation; however, in this context, cysteine blockage of HpHsp60 was found to strengthen this pro-inflammatory response. CM-modified HpHsp60 exacerbated both NF-κB transcription factor/DNA binding activity and follow-up cytokine secretion in monocytic cells. This phenomenon may indicate the importance of HpHsp60 oligomerization to inflammation. With its correct structure, HpHsp60 should be able to moderate the host immune response and thus may facilitate *H. pylori* survival and persistent infection within the gastric microenvironment.

In this study, we have shown that the oligomerization of HpHsp60 is quite different from that of homologs in other bacterial species. HpHsp60 preferentially forms dimers and tetramers rather than the heptamers of *E. coli* Hsp60 and this change in oligomeric status might affect the protein's functioning in terms of activating NF-κB signaling and inducing the release of pro-inflammatory cytokines.

Acknowledgments

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