Helicobacter pylori infection is a crucial factor in the pathogenesis of several digestive disorders, including peptic ulcers, chronic gastritis, and gastric cancer. Moreover, H. pylori induces disease-specific protein expression in gastric epithelial cells. The aim of the present study was to characterize proteins differentially expressed in H. pylori-infected gastric epithelial AGS cells. An in vitro model was established using a multiplicity of infection of 100 and evaluating the effectiveness of H. pylori infection by functional analyses. Changes in protein patterns were identified using a proteomic approach consisting of two-dimensional differential fluorescence difference gel electrophoresis and mass spectrometry. The expression of many proteins was found to be altered, and 28 of these were identified and classified as protein synthesis- and folding-related proteins, cytoskeleton proteins, metabolic enzymes, transcription- and translation-related proteins, angiogenesis/metastasis-related proteins, cell communication/signal transduction-related proteins, or others (oxygen-regulated protein and oncoprotein). The expression profiles of eight of these proteins, laminin γ-1 chain precursor, valosin-containing protein, heat shock 70-kDa protein, mitochondrial matrix protein P1, FK506-binding protein 4, T-complex protein 1, enolase α, and 14-3-3 β were further examined in cancerous and paired surrounding normal tissues by immunoblot assay and immunohistochemical staining to identify molecular targets that may be involved in the pathogenesis of H. pylori-induced gastric diseases. On the basis of our results, valosin-containing protein, mitochondrial matrix protein P1, T-complex protein 1, enolase α, and 14-3-3 β may play a crucial role in H. pylori-induced gastric carcinogenesis by mediating antiapoptotic and proliferative responses. Molecular & Cellular Proteomics 5:702–713, 2006.

From the †Graduate Institute of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei 100, Taiwan, §Department of Molecular Medicine, China Medical University Hospital, Taichung 404, Taiwan, ¶Department of Proteomics, Biomedical Engineering Center, Industrial Technology Research Institute, Hsinchu 310, Taiwan, and Departments of ¶¶Internal Medicine and **Medical Genetics, National Taiwan University Hospital, Taipei 100, Taiwan

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1 The abbreviations used are: IL-8, interleukin 8; m.o.i., multiplicity of infection; HGFR, hepatocyte growth factor receptor; 2-D DIGE, two-dimensional differential fluorescence DIGE; nano-LC-MS/MS, nano-flow LC-MS/MS; VCP, valosin-containing protein; MMP-P1, mitochondrial matrix protein P1; TCP1, T-complex protein 1; laminin γ-1, laminin γ-1 chain precursor; HSP, heat shock protein; KBP4, FK506-binding protein 4 (59 kDa); COX-2, cyclooxygenase-2; HRP, horseradish peroxidase; 2-DE, two-dimensional gel electrophoresis; NFκB, nuclear factor-κB; IκBα, inhibitor κBα; E1, ubiquitin-activating enzyme.
unclear. Several studies have focused on the differential expression of genes induced by *H. pylori* infection. Microarray analysis of gene expression induced by *H. pylori* in AGS cells showed marked changes in ~200 genes, and the up-regulation of c-jun, jun-B, c-fos, and cyclin D1 was confirmed by RT-PCR analysis (14). Another microarray study showed that a CagA-positive *H. pylori* strain induced the expression of cell adhesion-related genes in AGS cells (15) that may be related to *H. pylori*-associated gastric carcinogenesis. Moreover, the expression of genes encoding growth factors, cytokines/chemokines, such as IL-8, and antiapoptotic proteins, such as Bax and p53, has been found to be up-regulated by *H. pylori* (16). However, changes in the protein profile of host cells infected with *H. pylori* have not been investigated extensively.

In the present study, an *in vitro* model was established to characterize proteins differentially expressed in *H. pylori*-infected gastric epithelial cells that may play important roles in the pathological mechanism of *H. pylori*-induced gastric diseases. In an initial step, induction of the hummingbird phenotype, IL-8 release, and COX-2 protein expression was used to indicate the effectiveness of *H. pylori* infection of gastric epithelial cell, and then a fractionation method was applied to eliminate the contamination of bacterial proteins from the host proteome. Finally, a combination of two-dimensional fluorescence DIGE (2-D DIGE), which can separate thousands of proteins for quantitative comparison, and mass spectrometry was used to identify proteins showing altered expression. This is the first study using subcellular and functional proteomics to profile protein changes in AGS cells before and after *H. pylori* infection.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strain and Growth Conditions**—The *H. pylori* strain used in this study was isolated from human gastric biopsy samples obtained from patients with gastric cancer at the National Taiwan University Hospital, Taipei, Taiwan. The bacteria were inoculated onto Columbia agar containing 5% sheep blood (Invitrogen) and grown at 37 °C in a microaerophilic chamber (Don Whitley, West Yorkshire, UK) in 10% CO2, 5% O2, and 85% N2 (17).

**H. pylori Infection of AGS Cells**—The AGS human gastric epithelial cell line (gastric adenocarcinoma, ATCC CRL 1739) was obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in the presence of 100 units/ml penicillin-streptomycin at 37 °C in a humidified chamber in 5% CO2 (18). The cells were seeded at 1 × 106 cells/dish in 10-cm cell culture dishes, grown to 80% confluency, and then washed twice with PBS, pH 7.4, and the medium was replaced with antibiotic-free RPMI 1640 medium. Bacterial colonies were harvested and washed with PBS, and then the bacteria were resuspended in antibiotic-free RPMI 1640 medium. After determining their concentration from the *A*600, the bacteria were added to the cultured cells at an m.o.i. of 100.

**Induction of the Hummingbird Phenotype**—After infection for 4 h, the phenotype was analyzed by phase-contrast microscopy. Induction of the hummingbird phenotype was defined as more than 50% of the cells being elongated and scattered (Fig. 1).

**IL-8 ELISA**—After infection for 24 h, the supernatants from AGS cells incubated for 24 h in the absence or presence of *H. pylori* were assayed for IL-8 using ELISA kits (R&D Systems, Minneapolis, MN) in triplicate. Purified human recombinant IL-8 was used as standard. The minimum detectable IL-8 concentration was 10 pg/ml.

**COX-2 Immunoblot Analysis**—Control AGS cells or cells infected for 24 h with *H. pylori* were washed with ice-cold PBS containing 1 mM Na2VO4, and then the cells were scraped off on ice and lysed by sonication for 30 s on ice in 600 μl of lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris, pH 8.5). After centrifugation at 1,200 × g for 15 min at 4 °C, the protein concentration in the supernatant was determined using a 2-D Quant kit (Amersham Biosciences). Samples of protein (50 μg/lane) were separated by electrophoresis on 15% SDS gels and transferred onto a PVDF membrane (Millipore), which was then blocked for 1 h at 25 °C with skimmed milk. COX-2 was detected by incubation overnight at 4 °C with a polyclonal goat anti-COX-2 antibody (1:2,000, Santa Cruz Biotechnology) followed by incubation for 1 h at 25 °C with horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody (1:8,000, Sigma). After six washing steps, bound antibody was detected using ECL reagent (Amersham Biosciences).

**Preparation of Polyclonal Anti-ureaseA Antiserum**—The full-length gene for ureaseA from *H. pylori* was ligated into the expression vector pQE 30 (Qiagen, Chatsworth, CA), and the construct was used to transform *Escherichia coli* M15 (Qiagen). Cultures of transformed cells were grown to an *A*600 of 0.6, and then expression was induced by addition of 1 mM isopropyl β-d-thiogalactoside for 3 h. The recombinant His6-ureaseA fusion protein was purified using a Ni2+ chelate affinity column (19). The purified protein showed a single band on SDS-PAGE and was identified by mass spectrometry.

A female New Zealand White rabbit was injected intradermally at 20 sites with a total of 500 μg of recombinant ureaseA in 1 ml of PBS emulsified with 1 ml of Freund’s complete adjuvant (Sigma). Boosters of 500 μg in 1 ml of PBS emulsified with 1 ml of Freund’s incomplete adjuvant (Sigma) were given intradermally at weeks 3 and 6, then the rabbit was bled 10 days after the last boost, and the serum was used for immunoblotting experiments.

**Cell Fractionation and Immunoblotting**—All procedures were performed at 4 °C. Cell fractionation was performed according to Stein et al. (20) with some modification. Non-infected and *H. pylori*-infected cells were pelleted for 5 min at 200 × g, resuspended in 600 μl of homogenization buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, and 0.5 mM EDTA), and then lysed by 10 cycles of slow aspiration into a 1-ml syringe and vigorous expulsion. The lysate was centrifuged for 10 min at 1,500 × g, and the supernatant was transferred to another centrifuge tube. The pellet contained the bacteria, unlysed cells, and the cytoskeletal fraction. The supernatant was then centrifuged for 1 h at 20,000 × g; the resulting supernatant contained the host cell cytosolic fraction, and the pellet contained the host cell membranes. The different fractions were analyzed by immunoblotting using mouse antibodies against β-actin (1:50,000, BD Pharmingen) or rabbit antibodies against hepatocyte growth factor receptor (HGFR) (1:500, Santa Cruz Biotechnology) or ureaseA (1:50,000). The secondary antibodies were HRP-conjugated anti-mouse IgG antibody or HRP-conjugated anti-rabbit IgG antibody (both 1:2,000, Jackson ImmunoResearch Laboratories, Inc.). Blots were processed as described above.

**2-D DIGE**—The cell lysate was labeled according to the manufacturer’s instructions (Amersham Biosciences). Briefly 50 μg of lysate was labeled with 400 pmol of cyanine dye (Cy2, Cy3, and Cy5; Amersham Biosciences) on ice for 30 min in the dark. The labeling reaction was stopped by the addition of 1 μl of a 10 mM L-lysine solution (Sigma) and left on ice for 10 min. Proteins extracted from *H. pylori*-infected and non-infected cells were labeled with Cy3 and Cy5, respectively, mixed with Cy2-labeled internal pooled standard and run in the same gel. The internal pooled standard sample was prepared by pooling 25 μg of proteins of each sample from *H. pylori*-infected and non-infected cells. Analysis was repeated with reciprocal labeling, and each labeling was repeated three times.
Isoelectric focusing was performed for 205,000–245,000 V-h using the IPGphor system (Amersham Biosciences). After focusing, the IPG strips were equilibrated for 15 min with gentle shaking in 10 ml of 50 mM Tris-HCl (pH 8.8), 6 M urea, 50% (w/v) glycerol, 2% (w/v) SDS, and 0.01% (w/v) bromophenol blue. The second dimension electrophoresis was performed on 10% SDS gels in a Hoefer DALTTM tank (Amersham Biosciences) for 5 h at 20 mA/gel. For preparative gel for MS analysis, 1 mg of internal pooled standard proteins was used, and the gel was stained with Cooamassie Brilliant Blue G-250.

Images were acquired using a fluorescence scanner at appropriate wavelengths for Cy2, Cy3, and Cy5 dyes (Typhoon 9400TM, Amersham Biosciences). Images were preprocessed using the ImageQuantTM software (Amersham Biosciences). Intragel spot detection and intergel matching were carried out with the differential in-gel analysis mode and biological variation analysis mode of DeCyder software (Amersham Biosciences), respectively. Ratios of differentially expressed proteins were calculated using DeCyder software, and only those spots with a 1.5-fold increase/decrease were defined as altered.

**Mass Spectrometry and Protein Identification**—Protein spots were excised from preparative gels with a pipette tip, destained, and in-gel digested as follows. Briefly the spots were destained using 50 mM NH4HCO3 in 50% acetonitrile and dried in a SpeedVac concentrator. The protein was then digested by incubation overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI) in 50 mM NH4HCO3, pH 7.8. The resulting peptides were extracted sequentially with 100 μL of 1% TFA and 100 μL of 0.1% TFA, 60% acetonitrile, and the combined extracts were lyophilized, resuspended in 10 μL of 0.1% TFA, and desalted using a ZipTip C18 column (Millipore).

Tryptic digest samples were loaded onto a C18 preconcentration column (5 mm × 250 μm, PepMap C18, LC Packings, Amsterdam, Netherlands), and the peptides were separated on a reverse phase nanocolumn (15 cm × 75 μm, LC Packings). Peptide separation was performed using a 60-min linear gradient of 5–95% acetonitrile in 0.5% formic acid at a flow rate of 200 nl/min. Peptides were characterized using a Qstar XL Q-TOF mass spectrometer (Applied Biosystems). Proteins were identified by automated searching (Mascot Daemon, Matrix Science) against the National Center for Biotechnology Information (NCBI) protein database.

**2-DE Immunoblot Analysis**—After 2-DE of 500 μg of fractionated proteins, immunoblot analyses were performed as described above. The primary antibodies used were mouse anti-laminin γ-1 chain (laminin γ-1 antibody) (1:200), rabbit anti-valosin-containing protein (VCP) antibody (1:200), rabbit anti-enolase α antibody (1:2,000), and goat anti-14-3-3 β antibody (1:500) (all from Santa Cruz Biotechnology) and rabbit anti-heat shock 70-kDa protein (HSP70) antibody (1:2,000), mouse anti-mitochondrial matrix protein P1 (MMP-P1) antibody (1:2,000), mouse anti-FK506-binding protein 4 (FKBP4) antibody (1:200), and rat anti-T-complex protein 1 (TCP1) antibody (1:1,000) (all from Stressgen, British Columbia, Canada). The secondary antibodies used were HRP-conjugated anti-mouse IgG antibody, HRP-conjugated anti-rabbit IgG antibody (both 1:2,000, Jackson Immunoresearch Laboratories, Inc.), HRP-conjugated anti-goat IgG antibody (1:8,000, Sigma), and HRP-conjugated anti-rat IgG antibody (1:2,000, Stressgen). Membranes were developed with ECL reagent (Amersham Biosciences) or detected by 2-amino-9-ethylcarbazole as substrate system.

**Clinical Specimens**—Pairs of cancerous and corresponding noncancerous gastric tissues for immunoblot assay and immunohistochemistry were obtained from patients diagnosed with gastric adenocarcinoma who underwent surgical gastric resection at the Department of Pathology, National Taiwan University Hospital, and Department of Pathology, Changhua Christian Hospital, between May 2000 and January 2002. The age of the patients ranged from 54 to 81 years old with a mean of 70.6 years old. Clinically two cases were stage II, three cases were stage IIIA, and five cases were stage IV, respectively. Pathologically one case was well differentiated adenocarcinoma, five cases were moderately differentiated adenocarcinoma, and four cases were poorly differentiated adenocarcinoma. These patients all were positive for H. pylori infection determined by culture and/or histological examination of the gastric biopsy specimens. Written informed consent was obtained from all patients before surgery.

**Immunoblot Analysis of Cancerous and Non-cancerous Tissues**—The gastric tissue samples were ground in a mortar filled with liquid nitrogen and then extracted for 16 h at 4 °C with constant stirring with 50 mM phosphate buffer, pH 7.0, containing 0.2 mM DTT and 1 mM PMSF. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant was dialyzed for 48 h at 4 °C against 10 mM phosphate buffer, pH 7.0, and lyophilized, and then 50-μg samples were subjected to electrophoresis on SDS gels and immunoblotting using ECL reagent as described above. After exposure to x-ray film and analysis by densitometry, differences in expression between the cancerous and corresponding non-cancerous tissues were analyzed using the paired Student’s t test with a p value of < 0.05 being considered statistically significant.

**Immunohistochemistry**—Paraffin-embedded tissue sections (4 μm thick) on poly-L-lysine-coated slides were deparaffinized, treated with 3% H2O2 in methanol for 30 min to quench endogenous peroxidase activity, rehydrated with an alcohol gradient, and rinsed with PBS. The sections were then microwaved in citrate buffer, pH 6.1, for 20 min for antigen retrieval. Nonspecific binding was blocked by incubation for 1 h at room temperature with PBS containing 5% skimmed milk, and then the sections were rinsed with PBS and incubated for 30 min at room temperature with primary antibodies against laminin γ-1, VCP, HSP70, MMP-P1, FKBP4, TCP1, enolase α, or 14-3-3 β (all diluted 1:50 in PBS) and for 30 min at room temperature with anti-mouse, anti-rabbit, anti-goat, or anti-rat IgG HRP/Fab polymer conjugate (Zymed Laboratories Inc., PicTure polymer kit) and then washed three times with PBS. Bound antibody was visualized using diaminobenzidine chromatogen (Merck) and counterstained with hematoxylin (21). Sections of cancer tissue overexpressing the target protein were used as positive controls, and normal serum instead of primary antibody was used in negative controls.

**RESULTS**

**Induction of the Scattering Phenotype, IL-8 Release, and COX-2 Protein Expression in AGS Cells Induced by H. pylori Infection**—To study H. pylori pathogenesis in vitro, AGS gastric epithelial cells were infected with H. pylori, and infection was monitored by the appearance of the scattering hummingbird phenotype. This phenotype, characterized by spreading and elongation of the host cells and loss of cell-cell contact, was seen using an m.o.i. of 100 as in a previous report (22). Morphological changes in AGS cells were seen after 4 h of infection when more than 50% of the cells exhibited the hummingbird phenotype (Fig. 1A). Previous reports have demonstrated that H. pylori can also induce both IL-8 release and COX-2 expression (18, 23) in vitro. ELISA showed that IL-8 levels in the medium were 34.4 ± 12.9 pg/ml for noninfected cells and 1,118.3 ± 47.4 pg/ml for cells infected with H. pylori for 24 h (Fig. 1B), whereas immunoblot analysis showed that COX-2 expression was induced in H. pylori-infected cells after 24 h (Fig. 1C). Our results therefore
showed that phenotypic changes, IL-8 release, and COX-2 protein expression were induced in *H. pylori*-infected AGS cells, demonstrating the effectiveness of *H. pylori* infection of gastric epithelial cells and suggesting that the response in gastric epithelial cells might be altered during *H. pylori* pathogenesis.

**Cell Fractionation of Non-infected and *H. pylori*-infected AGS Cells**—To avoid interference of bacterial proteins with the proteome analysis, cell fractionation was performed to remove whole bacteria and the AGS cell membrane fraction containing the adherent *H. pylori*. HGFR was used as a marker for the membrane fraction. Our data showed that HGFR was detected in both the membrane and unlysed cell plus bacteria fractions but not in the cytosolic fraction (Fig. 2A). UreaseA, a major protein of *H. pylori* that is found both in the bacterial cytosol and associated with the outer bacterial membrane (24), was used as a control to show that the bacteria were not lysed during the fractionation procedure. Fig. 2B shows that ureaseA was absent in the host cytosol fraction, present at low amounts in the membrane fraction, present at high amounts in the bacteria plus cell pellet fraction of *H. pylori*-infected cells, and absent in all fractions of non-infected cells. β-Actin was used as a loading control because of its abundance in each fraction (Fig. 2C). This quality control analysis was applied before 2-DE and 2-DE immunoblot assays.

**Identification of Differentially Expressed Proteins Induced by *H. pylori* Infection**—The above experimental results confirmed that the method used resulted in effective *H. pylori*
infection of AGS cells. Protein expression was compared between cytosolic fraction of non-infected and *H. pylori*-infected AGS cells using DIGE technology with a mixed sample internal standard. 2-DE separation was initially performed using a pH range of 3–10 and 12.5% SDS gels. After several runs, we found that most differentially expressed proteins were larger than 30 kDa and focused in the range pH 4–7, so subsequent experiments were performed using pH 4–7 linear strips and 10% SDS-PAGE. As diagrammed in Fig. 3A, a pseudocolor map of superimposed DIGE images is presented (Cy3-labeled *H. pylori*-infected cell lysates and Cy5-labeled non-infected cell lysates). We also adopted a dye swapping strategy to avoid dye labeling bias; therefore, Cy3 and Cy5 dyes were interchangeable (data not shown).

The analysis of the expression profiles of AGS cells using DeCyder software resulted in 28 significantly and reproducibly regulated spots induced by *H. pylori* infection (*p* < 0.05, ratio > 1.5; Fig. 3B). To identify these differentially expressed proteins, the spots of interest were excised from the preparative gel, and in-gel digestion and nano-LC-MS/MS spectrometry were performed for protein identification (Table I and Supplemental Table I). 28 proteins that were up- or down-regulated after *H. pylori* infection were identified, and seven were categorized as protein synthesis- and folding-related proteins, six were categorized as cytoskeleton proteins, six were categorized as metabolic enzymes, three were categorized as transcription- and translation-related proteins, two were categorized as angiogenesis/metastasis-related proteins, two were categorized as cell communication and signal transduction proteins, one was categorized as oxygen-regulated protein, and one was categorized as fusion oncoprotein by bioinformatics ontology. VCL, villin 2, T-complex protein 1, mitochondrial matrix protein P1, FKBP4, enolase, and TPM4-ALK fusion oncoprotein type 2 existed as more than one isoform in the proteome profile, indicating these proteins underwent post-translational modifications. Most proteins, except 150-kDa oxygen-regulated protein, VCL, and heat shock protein 90 kDa, were up-regulated after *H. pylori* infection.

2-DE Immunoblot Analysis of the Eight Cancer-associated Proteins—Of the proteins showing an increase in expression of more than 1.5-fold after *H. pylori* infection, eight were selected for further analysis; these were laminin-1, VCP, HSP70, MMP-P1, FKBP4, TCP1, enolase α, and 14-3-3 β, all

![Fig. 2. Cell fractionation of non-infected and *H. pylori*-infected AGS cells. Bacteria and unlysed cells were removed, host cytosolic and membrane fractions were prepared, and then all three fractions were analyzed by electrophoresis on 10% SDS gels and immunoblotting using antibodies against HGFR (145 kDa) (A), ureaseA (31 kDa) (B), or β-actin (43 kDa) (C).](image1)

![Fig. 3. 2-D DIGE analysis of alterations in the cytosolic fraction of AGS cells induced by *H. pylori* infection. *H. pylori*-infected cell lysates were labeled with Cy3 (green), non-infected cell lysates were labeled with Cy5 (red), and internal pooled standard lysates were labeled with Cy2. The first dimension was performed on 13-cm IPG strips, pH 4–7, and proteins were further separated by SDS-PAGE (10%). A, overlaid 2-D gel images of *H. pylori*-infected (green) and non-infected (red) protein spot patterns. B, representative 2-D image of pooled standard proteins. Proteins showing altered expression after *H. pylori* infection and identified by nano-LC-MS/MS spectrometry are numbered and shown in Table I, and the unique peptide list is shown in Supplemental Table I.](image2)
of which have been reported to be correlated with gastric or non-gastric cancer (25–32). The identity and expression profiles of these proteins were confirmed using 2-DE immunoblot analysis (Fig. 4). The greatest changes were seen for laminin α1, VCP, HSP70, MMP-P1, and 14-3-3 β, whereas moderate changes were seen for FKBP4, TCP1, and enolase α.

**Expression of the Eight Cancer-associated Proteins in Cancerous and Surrounding Normal Gastric Tissues**—The expression of these eight proteins was evaluated in clinical specimens. Ten pairs of cancerous and surrounding normal tissues from gastric adenocarcinoma patients were analyzed by SDS gel electrophoresis and immunoblotting, and the results were quantified using ImageMaster™ Platinum version 5.0 software. Spots in which the expression in the cancerous tissue was more than 2-fold higher than in the “paired” surrounding normal tissues were considered “increased spots.” Increased spots were seen in nine of the 10 paired samples for laminin α1, six for VCP, seven for HSP70, seven for MMP-P1, 10 for FKBP4, six for TCP1, 10 for enolase α, and 10 for 14-3-3 β (Fig. 5). The increase in expression in cancerous tissues was statistically significant for all eight proteins, suggesting their overexpression may be correlated with gastric carcinogenesis.

**Immunohistochemical Staining for the Eight Cancer-associated Proteins in Cancerous and Surrounding Normal Gastric Tissues**—To confirm the expression levels of these eight proteins and compare the levels in the cancerous tissues and surrounding normal tissues, immunohistochemical staining was performed on tissue microarrays. The staining intensity was scored using a semi-quantitative method, and the results were analyzed using ImageJ software. The staining patterns were consistent with the immunoblotting data, confirming the overexpression of these proteins in cancerous tissues.

### Table I

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<td>Oxygen-regulated protein (150 kDa)</td>
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<td>18</td>
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<tr>
<td>27</td>
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<td>29</td>
<td>2.03</td>
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</table>
paired surrounding normal tissues, immunohistochemical staining was performed. The results of histological staining of the cancerous and paired normal tissues are summarized in Table II. In total, 10 samples were evaluated. In the expression of VCP, nine of them had more abundant expression in gastric cancerous cells than in surrounding normal cells, and one of them had a similar amount of expression between gastric cancerous cells and normal cells. In the expression of TCP1, five of them had more abundant expression in gastric cancerous cells than in normal cells, four of them had a similar amount of expression between gastric cancerous cells and normal cells, but one of them had less abundant expression in gastric cancerous cells than in normal cells. In the expression of MMP-P1, five of them had more abundant expression in gastric cancerous cells than in normal cells, and five of them had a similar amount of expression between gastric cancerous cells and normal cells. In the expression of enolase α, nine of them had more abundant expression in gastric cancerous cells than in normal cells, and one of them had a similar amount of expression between gastric cancerous cells and normal cells.

Fig. 4. 2-DE immunoblot analysis (A) and three-dimensional fluorescence intensity profiles (B) of non-infected AGS cells and AGS cells infected for 24 h with H. pylori at an m.o.i. of 100. Protein identities and the expression profiles of the eight representative proteins were confirmed by 2-DE immunoblotting and nano-LC-MS/MS analysis, respectively.
FIG. 5. Expression of laminin γ-1, VCP, HSP70, TCP1, MMP-P1, FKBP4, enolase α, and 14-3-3 β in paired cancerous (T) and non-cancerous (N) gastric tissues. Protein samples (50 μg) from cancerous and corresponding normal tissue prepared as described under "Experimental Procedures" were electrophoresed on SDS gels and then immunoblotted using specific antibodies. The densitometric results for all paired samples (n = 10) for each protein were standardized to those for β-actin. The circles show the scatter plot, and the squares indicate the mean ± S.E. for all 10 samples. Differences in expression were analyzed using the paired Student’s t test. \( p < 0.05 \) is considered statistically significant.
normal cells. In the expression of 14-3-3 β, three of them had more abundant expression in gastric cancerous cells than in normal cells, and seven of them had a similar amount of expression between gastric cancerous cells and normal cells (Fig. 6). Most cases had similar expression levels of laminin γ-1, HSP70, and FKBP4 proteins between gastric cancerous cells and paired normal cells (data not shown).

**DISCUSSION**

In the present study, we established an in vitro cell model to investigate the pathological mechanism of gastric diseases, especially gastric cancer, caused by *H. pylori*. The AGS cell is a transformed cell line but is similar to normal gastric cells (33), so the changes in protein expression in AGS cells induced by *H. pylori* infection might be expected to resemble the transcriptional or translational activation in normal gastric epithelium.

In our study, we confirmed the effectiveness of *H. pylori* infection of AGS cells by three different functional analyses, namely the induction of the hummingbird phenotype, IL-8 release, and COX-2 expression. The scattering hummingbird phenotype has been described as indicative of the translocation of CagA, the most important virulence factor of *H. pylori*, into epithelial cells, inducing host cellular growth changes (10, 34). In addition, translocated CagA activates the nuclear factor-κB (NF-κB) signaling pathway and leads to increased production of cytokines, such as IL-8. Increased mucosal expression of IL-8 may result in a greater degree of gastritis, ultimately predisposing to the development of gastric carcinoma (35, 36). COX-2 is the inducible key enzyme of arachidonic acid metabolism and contributes to the pathogenesis of gastroduodenal ulcers and gastric cancer. COX-2 expression induced in the stomach by *H. pylori* infection is a relatively early event during carcinogenesis (37). The results showing the phenotype change, IL-8 release, and COX-2 protein expression suggest that the host response, including inflammation, hyperproliferation, and an antiapoptotic response, could be evoked in AGS cells by *H. pylori* infection.

Cell fractionation (20) was successfully used to remove the *H. pylori* proteins that otherwise severely interfered with protein quantification of 2-DE profiles. This is the first report of a cell fractionation method being used to examine differential expression induced by *H. pylori* either at the gene or protein level.

The 2-D DIGE technology combined with mass spectrometry is a powerful tool for analyzing protein changes in gastric epithelial cells associated with *H. pylori* pathogenesis. Many proteins showing altered expression were found in this study, but only those with important functions and showing major changes were analyzed further. We identified 28 proteins that showed variations in concentration following *H. pylori* infection. These were classified as protein synthesis- and folding-related proteins, cytoskeleton proteins, metabolic enzymes, transcription- and translation-related proteins, angiogenesis/metastasis-related proteins, cell communication/signal transduction-related proteins, oxygen-regulated protein, and oncoprotein on the basis of their molecular functions. The expression of eight of these was investigated by immunoblotting and immunohistochemistry in paired samples of clinical cancerous and corresponding normal gastric tissues. The characteristics and results for each of these proteins are discussed below.

**Valosin-containing Protein**—The most interesting protein showing changes in this study was VCP. VCP is a member of the ATPase family associated with various cellular activities and is involved in the ubiquitin-dependent proteasome degradation of inhibitor κBα (IκBα), which is an inhibitor of NFκB. Cell lines transfected with VCP show constant activation of NFκB, rapid degradation of phosphorylated IκBα, decreased apoptosis, and increased metastatic potential (38). Furthermore, a correlation between VCP expression, metastasis, and prognosis has been reported in human hepatocellular carcinoma (39), pancreatic ductal adenocarcinoma (26), and gastric carcinoma (40).

**T-complex Protein 1**—TCP1 is a hetero-oligomeric molecular chaperone that assists in the folding of actin, tubulin, and other cytosolic proteins. TCP1 expression is closely correlated with the growth rate of mammalian cultured cells and is increased in human hepatocellular and colonic carcinoma. TCP1 expression is up-regulated in rapidly proliferating tumor cells, thus helping in the production of the proteins required for growth, and may serve as a useful tumor marker in hepatocellular and colonic carcinoma (30).

**Laminin γ-1 Chain Precursor**—Laminin-1, a heterotrimer of α-1, β-1, and γ-1 chains, has multiple biological activities, including promoting cell adhesion, migration, differentiation, neurite outgrowth, and tumor cell malignancy. Laminin-1 induces production of collagenase IV, urokinase-type plasminogen activator, and the 92-kDa matrix metalloproteinase (MMP-9) (41) *in vitro*. In addition, laminin-1 enhances the metastatic phenotype of tumor cells *in vitro* (42).

**Mitochondrial Matrix Protein P1 and Heat Shock 70-kDa Protein**—Heat shock proteins (HSPs) are highly conserved...
molecules that act as chaperones involved in the correct folding of newly synthesized proteins. Several families of HSPs, such as the small HSP (HSP27) family, HSP40 family, HSP60/chaperonin family, HSP70 family, and HSP90 family, have been identified in mammalian cells (43). Mitochondrial matrix protein P1 belongs to the HSP60/chaperonin family and is found specifically in mitochondria where its major function is protein chaperoning and folding. Aberrant HSP60 expression has been associated with autoimmune diseases (44). HSP70, a major member of the HSP70 family, regulates a wide range of protein-associated activities, including protein denaturation-renaturation, folding, transport-translocation, activation, and secretion. HSP70 expression increases after transformation by oncogenes (45), and elevated HSP70 levels protect cells from apoptotic death (46). Increased expression of HSP60 and HSP70 also occurs in several malignant tumors, such as osteosarcoma, and prostate cancer (47, 48).

FK506-binding Protein 4—FKBP4 is a member of the immunophilin protein family, which plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking. FKBP4 is a cis-trans prolyl isomerase that binds the immunosuppressants FK506 and rapamycin. It interacts with interferon regulatory factor-4 and plays an important role in immunoregulatory gene expression in B and T lymphocytes (49). FKBP4 can also associate with two heat shock proteins (HSP90 and HSP70) and thus may play a role in the intracellular trafficking of hetero-oligomeric forms of the steroid hormone receptors (50). Overexpression of FKBP4 found in invasive ovarian tumors indicates it may serve as a new biomarker and therapeutic target (29).

Enolase α—Enolase α, a key glycolytic enzyme, serves as a plasminogen receptor on the surface of a variety of hematopoietic, epithelial, and endothelial cells. It is involved in various pathological events such as tissue remodeling and the spread of transformed tumor cells induced by plasminogen activation (51). It has also been described as a stress protein induced by hypoxia, and overexpression of enolase α has been reported in several cancer cell lines and autoimmune disease. Furthermore some evidence indicates a relation between enolase α expression and the progression of tumors, such as lung cancer and hepatocellular carcinoma (52, 31).

14-3-3—14-3-3 proteins play crucial roles in regulating multiple cellular processes including the maintenance of cell cycle checkpoints and DNA repair, signal transduction, and the prevention of apoptosis. In mammalian cells, seven isoforms (β, γ, ζ, α, ε, η, and η) have been identified, and each of these seems to have distinct tissue localizations and isoform-specific functions. It has been reported that 14-3-3 β can bind integrin, testicular protein kinase 1, and Wee1 to regulate cell spreading and $G_2-M$ transition (53). Notably overexpression of 14-3-3 β has a role in the proliferation and oncogenic transformation of NIH 3T3 cells (54).

The immunoblot analyses showed that these eight proteins were overexpressed in more than 60% of the cancerous tissue samples compared with the corresponding normal tissue. However, only VCP, MMP-P1, TCP1, enolase α, and 14-3-3 β showed stronger immunoreactivity in tumor tissues. These differences may be due to the greater sensitivity of the ECL method used for immunobLOTS compared with the staining methods used for immunohistochemistry. To clarify the significance of the overexpression of these candidate tar-
gets, further functional analyses and an increased sample size are needed.

Overall VCP, proteasome 26 S ATPase subunits 1 and 2, and ubiquitin-activating enzyme E1 were all found to be over-expressed after \textit{H. pylori} infection, suggesting that the NF-κB signaling pathway, which has been shown to be the main cellular event evoked by \textit{H. pylori}, was constantly activated and induced an antiapoptotic effect mediated by the VCP-proteasome degradation machinery. Previously reactive oxygen species were suggested as a major signal of NF-κB activation in \textit{H. pylori}-infected AGS cells (55). Up-regulation of 14-3-3 protein and enolase \( \alpha \) in \textit{H. pylori}-infected AGS cells and gastric cancer tissues demonstrates that the involvement of oxidative stress in \textit{H. pylori}-induced carcinogenesis. In addition, the overexpression of TCP1 seen in \textit{H. pylori}-infected gastric cells may promote cell proliferation, whereas the increase in the chaperones, HSPs and FKBP4, may help in the production of the extra proteins needed. Laminin-1 and its receptor laminin-binding protein were both up-regulated in \textit{H. pylori}-infected gastric cells, demonstrating that \textit{H. pylori} may trigger laminin signaling to promote cell metastasis by an as yet unknown mechanism. These eight proteins may induce an antiapoptotic response and promote proliferation after \textit{H. pylori} infection and thus be novel molecular targets, which may play a crucial role in the \textit{H. pylori} pathological mechanism.

In conclusion, an increased knowledge of changes in the protein profile induced by \textit{H. pylori} infection is important in understanding the pathological mechanism of this bacterium. This study not only used a typical proteomic approach to identify differentially expressed proteins but also adopted functional analyses before 2-DE map comparison and evaluated the expression of proteins of interest in clinical samples. Further detailed analysis of these novel target proteins in investigating the mechanism of \textit{H. pylori}-induced gastric diseases in AGS cell lines is now in progress. The expression of the other \textit{H. pylori}-regulated proteins identified in this study will also be evaluated in clinical samples.

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\[ \text{The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.} \]

† To whom correspondence should be addressed: Graduate Inst. of Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Rd., Taipei 100, Taiwan. Tel.: 886-2-23123456 (ext. 8212); Fax: 886-2-23958814; E-mail: lupin@ha.mc.ntu.edu.tw.

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