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Gene Expression Profiling in AGS Cells Stimulated with *Helicobacter pylori* Isogenic Strains (*cagA* Positive or *cagA* Negative)

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To study host response to CagA, human gastric cancer cell line AGS was infected with a *Helicobacter pylori* type I wild-type or isogenic *cagA*-negative mutant. Differentially expressed genes were identified using cDNA array technology. By Northern blotting, downregulation of focal adhesion kinase and upregulation of LIM kinase mRNA in the presence of CagA were clearly verified. Furthermore, upregulation of LIM kinase, macrophage inflammatory protein-2, c-*myc*, and bone morphogenetic protein-1 and downregulation of transcription factor Y-box binding protein-1 and focal adhesion kinase mRNA in response to *H. pylori* type I infection compared to the uninfected control could be shown by Northern blotting. Hence, these findings identified new targets for further functional studies on *H. pylori*-associated pathogenesis.

Helicobacter pylori type I strains exclusively harbor the multigene 40-kb *cag* pathogenicity island (PAI) associated with increased bacterial virulence contributing to a more severe inflammatory response in the host mucosa (5). Based on sequence homology, a putative type IV secretion apparatus encoded by multiple genes of the *cag* PAI has been described (5). This locus is required for the induction of interleukin (IL)-8 through an NF-κB-dependent pathway in gastric epithelial cells (5, 24). Furthermore, cytoskeletal reorganization, tyrosine phosphorylation of host proteins (23), and differential activation of MAP kinases (12, 14) occur in gastric epithelial cells after attachment of type I strains.

The *cagA* gene serves as a marker for the *cag* PAI, encoding an immunodominant size-variant protein of unknown physiologic function (7). Recently, it was shown that CagA is translocated into gastric epithelial cells via the putative type IV secretion system and subsequently becomes tyrosine phosphorylated (3, 16, 22, 25). However, the mode(s) of CagA action in the epithelial cell still needs to be elucidated.

In this study, cDNA array technology was used to investigate the role of CagA in host gene expression. cDNA expression arrays were screened in parallel to detect alterations in gene expression of AGS cells stimulated with an isogenic pair of *H. pylori* type I strains, a wild-type and a *cagA*-negative isogenic mutant. Subsequently, differentially expressed genes identified were subjected to further analysis by Northern blotting.

***H. pylori* growth and infection of AGS cells.** *H. pylori* strains were routinely cultured for 48 h on *H. pylori* selective agar (Biotest Laboratories) in a microaerobic atmosphere at 37°C. The human gastric epithelial adenocarcinoma cell line AGS (ATCC CRL 1739) was grown in RPMI 1640 supplemented with 4 mM L-glutamine and 10% fetal calf serum (Life Technologies Inc., Rockville, Md.) at 37°C in a humidified atmosphere of 5% CO_2. Prior to infection, viability of *H. pylori* cultures was routinely assessed by phase contrast microscopy. A monolayer of AGS cells (10^7) grown to 80% confluency was cocultured with *H. pylori* at a multiplicity of infection of 100 in culture medium for 4.5 h. As CagA translocation has been shown to occur 30 min after infection but is at its maximum in a time range of about 4 to 5 h (3, 15) the time point of 4.5 h chosen in our study seemed to be appropriate to investigate alterations in gene expression which may primarily reflect events early in host response to CagA.

cDNA array analysis. *H. pylori* wild-type P12 (*cag* PAI^+) and the corresponding *cagA*-negative isogenic mutant P17 derived by insertional mutagenesis (21) were used for stimulation. The absence of CagA protein in P17 was confirmed by Western blotting (results not shown). Total cellular RNA was extracted by the guanidinium thiocyanate single-step method (6) at 4.5 h postinfection and treated with RQ1 DNase (0.5 U/μg of RNA) (Promega, Madison, Wis.). Efficient *H. pylori* infection was controlled by detecting relative differences in IL-8 expression using semiquantitative reverse transcription (RT)-PCR. Briefly, cDNA was prepared from 5 μg of each preparation of total RNA dedicated to array analysis using Moloney murine leukemia virus reverse transcriptase (1 U/μg) (Promega, Madison, Wis.). Subsequent PCR was carried out under standard conditions using oligonucleotides specific for IL-8 (Table 1). For normalization, β-actin RT-PCR was performed. P12 and P17 were shown to induce IL-8 in contrast to uninfected control and no significant differences in IL-8 mRNA levels could be found between the two samples (data not shown).

For array analysis, poly$(A)^+$ RNA was prepared from total RNA by oligo(dT)-latex bead chromatography (Qiagen, Hilden, Germany). Atlas human cDNA expression arrays (Clontech, Palo Alto, Calif.) were used representing a broad range of 588 arrayed human cDNAs and housekeeping controls spotted in duplicate. The array is arranged into six sections of 98 genes each, representing the following functional groups: cell cycle regulator genes and oncogenes (region A), stress response and intracellular signaling-related genes (B),

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TABLE 1. Target-specific primer sequences for probe generation by PCR

GenBank accession no.	Name	Primer 1 (forward)	Primer 2 (reverse)	Positions	Transcript size (bp)
M22488	BMP-1	TCCTGGATACCATTGTCC	AGATGGCTTCGTAGACTG	874–1333	2,487
V00568	c-*myc*	TGCAGCCGTATTTCTACTG	CCTCATCTTCTTGTTCCTC	614–1337	2,121
L13616	FAK	GGTCATCTGGGAAGCCTTG	GAGGGTAGCAAGACGTGCTC	2879–3422	3,791
D26309	LIMK	CATCTCATGGCAAGCGTG	GGCTGAGTCTTCTCGTCCAC	607–1570	3,262
X53799	MIP2-α	GAATTCACCTCAAGAACATC	CCTAAGTGATGCTCAAAC	194–501	1,081
M83234	4B-1	CATCAACAGGAATGACAC	GCGTCTATAATGGTTACG	447–667	1,468
M28130	IL-8[a]	ATGACTTCCAAGCTGGCCGTGGCT	TCTCAGCCCTCTTCAAAAACTTCTC	1584–1873	5,191
NM001101	β-Actin[b]	CATGTACGTTGCTATCCAGGCTGTG	GAAGGTAGTTTCGTGGATGCCACAG	466–916	1,793
M33197	GAPDH[c]	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC	212–812	1,268

[a] Primer pair purchased from Clontech.
[b] Primer pair generously provided by R. Mader (Vienna, Austria).
[c] Primer pair purchased from Stratagene (La Jolla, Calif.).

apoptosis-related genes (C), DNA repair and recombination genes (D), transcription factors (E), and cell adhesion molecules and genes involved in cell-cell communication (F). Poly(A)$^+$ RNA (1 μg) was reverse transcribed into ^{32}P-labeled first-strand cDNA according to the manufacturer's instructions. Equal amounts of cDNA (10^6 cpm/μl) were hybridized overnight at 65°C to the arrays. Following the high-stringency washes, analysis of hybridization patterns was performed by autoradiography (Fig. 1) and scanning densitometry using the ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). Several film exposures were scanned to ensure that signal intensities were within the linear range of the film. Control spots on arrays were negative for genomic DNA contamination.

Ratios of signal intensities between potential differentially expressed genes were calculated. The constitutively expressed genes produced comparable signal intensities on both membranes which did not change by more than 0.25-fold between the arrays. After background reduction, an average of these values was used for normalization of the arrays to correct probe variation between signals to be validated. Subsequently, ratios of expression levels were calculated for each gene producing sufficient signal intensities to be analyzed (176 genes, 29.9%). Overall, 75 of these genes (42.6%) exhibited a more than 0.25-fold difference in abundance between the arrays. As no replicate was performed, a very stringent cutoff for significant up- or downregulation of genes was set to a 2.5-fold difference in transcript abundance to avoid false-positives. However, we are aware that by doing so some genes differentially regulated by small amounts may have been excluded from further analysis.

Figure 1 shows two representative regions of the array, including results from quantitative evaluation of the dots. Hence, three genes, LIM kinase (LIMK), bone morphogenetic protein 1 (BMP-1), and macrophage inflammatory protein 2α (MIP-2α), were identified to be upregulated. Three genes, focal adhesion kinaw (FAK), Y-box binding protein 1 (YB-1), and c-*myc*, appeared to be downregulated in AGS cells coincubated with P12 compared to those treated with the *cagA*-negative mutant strain (Table 2).

Confirmation of differential gene expression. Stimulation experiments were subsequently repeated at least three times as described, including other sets of isogenic strains: P12Δ*cagA*, a *cagA* knockout of P12 (16), as well as wild-type G27 (*cag* PAI$^+$) and *cagA* knockout strain G27Δ*cagA* (5, 22). As *cag* PAI-encoded factor CagE is critical for IL-8 induction and tyrosine phosphorylation, being a putative core protein of the predicted type IV system, a *cagE*-negative strain (10-B4) isogenic to G27 was also included (5). As expected, IL-8 expression was not induced in AGS cells stimulated with 10-B4 compared to stimulation with the G27 wild-type. In contrast, all other strains mentioned above induced IL-8 to a similar extent (results not shown).

For Northern analysis, DNase I-treated total RNA (35 μg) was separated on denaturing 1.2% agarose–formaldehyde gels and capillary transferred onto nylon membranes (Amersham Pharmacia Biotech). To generate the labeled probes, specific cloned cDNA fragments, amplified from the respective mRNAs (Table 1), were biotinylated by PCR using Pwo polymerase (Hoffmann-La Roche, Basel, Switzerland), and 3.5 nmol of dATP was replaced by biotin-14-dATP (0.4 mM; Gibco Life Technologies, Rockville, Md.) in the reaction mixture (0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$).

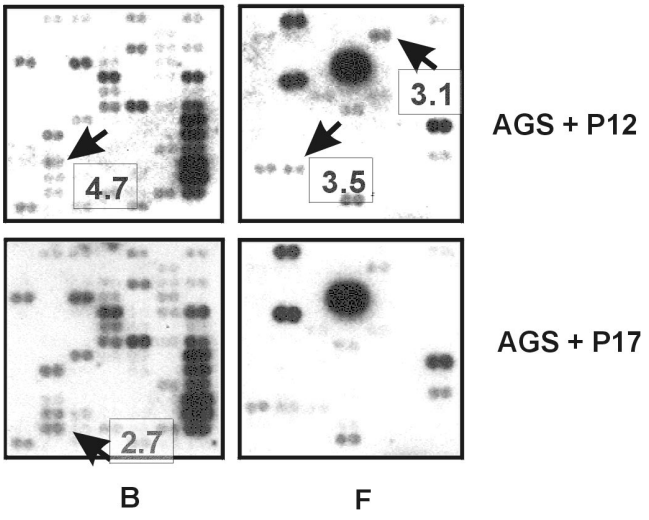

FIG. 1. Parallel fingerprints of AGS cells infected for 4.5 h with the P12 wild-type or its isogenic *cagA*-negative mutant, P17. Region B (stress response) and region F (cell-cell communication) of the human Atlas array are shown. Blots were exposed to X-ray film for 24 h. Arrows mark representative cDNA spots that showed significant differences in gene expression, and boxes indicate fold upregulation of the respective gene in AGS cells stimulated with the indicated *H. pylori* strain relative to the parallel approach.

TABLE 2. Names and GenBank accession numbers of upregulated transcripts after stimulation with P12 or P17 compared to the respective parallel approach

Stimulation	Upregulated transcripts (GenBank accession no.)	Fold stimulation	Functional role of gene
AGS + P12 (wild type)	LIMK (D26309)	4.7	Intracellular signal transduction, serine/threonine kinase
	BMP-1 (M22488)	3.5	Cell-cell communication, metalloproteinase
	MIP-2α (GRO beta) (X53799)	3.1	Cell-cell communication, cytokine (C-X-C subgroup)
AGS + P17 (*cagA* negative)	FAK (L13616)	2.7	Intracellular signal transduction, tyrosine kinase
	YB-1 (M83234)	2.6	Transcription factor
	myc proto-oncogene (V00568)	2.5	Oncogene

The membranes were prehybridized for 1 h in North2South hybridization buffer (Pierce, Rockford, Ill.) at 65°C and hybridized overnight to the cDNA probe (30 ng/ml of hybridization buffer). Four posthybridization washes were done at 20 min each at 65°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate–0.1% sodium dodecyl sulfate (SDS). Detection of cDNA/RNA hybrids was carried out using the North2South chemiluminescent detection system.

RNA loadings were normalized by blot hybridization to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe. Sizes of the transcripts were determined by relative migration versus eukaryotic 28S and 18S rRNAs. Blots were exposed to Kodak X-Omat films (Eastman Kodak, Rochester, N.Y.), and autoradiograms were quantified as described. Statistical analysis of quantitative evaluation was performed by comparing means using the one-way analysis of variance procedure and Bonferroni post hoc range test for multiple comparisons. Probability (P) values less than 0.05 were considered significant.

BMP-1, YB-1, MIP-2, and c-*myc* are differentially expressed in *H. pylori* type I infection. For these genes, results obtained by Northern blotting were not consistent with the differential data from array analysis, as no significant difference in expression between AGS cells stimulated with either the wild-types or the corresponding *cagA*-negative mutants was found. Importantly, all of these genes were found to be differentially regulated in response to *H. pylori* type I infection. In our opinion, the highly reproducible results ($P < 0.01$) obtained in triplicate series of Northern blot experiments (Fig. 2) are assumed to be more relevant than the data obtained from array analysis.

Transcription of BMP-1, a metalloproteinase which is putatively involved in morphogenesis and wound repair by altering cell-matrix interactions (10), was found to be upregulated following stimulation with the tested *H. pylori* wild-type G27 by up to 4.4-fold compared to the unstimulated control. Infection with P17 induced BMP-1 expression to similar levels as the wild-type P12. However, using P12Δ*cagA* or G27Δ*cagA* as the stimulus, a marked downregulation of the gene comparable to the AGS control could be found.

Furthermore, the transcript specific for YB-1 reproducibly appeared to be downregulated in all *H. pylori*-treated AGS populations, by up to the 3.2-fold (P12Δ*cagA*). Transcription factor YB-1 has been suggested to play a role in promoting cell proliferation, since Y-boxes are present in the promoters of several genes associated with cell division (26). It could therefore be speculated that a downregulation of YB-1 may be due to growth retardation of gastric epithelial cells occurring during *H. pylori* infection, as described (1). Our results provide first evidence that YB-1 and BMP-1 might be involved in host response to infection with *H. pylori*.

Our probe specific for MIP-2α (GRO beta), which is 95% homologous to the MIP-2β (GRO gamma) gene (8), detected both of the MIP-2-related mRNAs. Thus, the Northern blots revealed a profound upregulation of up to 45-fold of MIP-2, which has similar activities on neutrophils as IL-8, upon infection of AGS cells with all *H. pylori* type I strains. These results are in agreement with Yamaoka et al., who described an increase in GRO expression due to *H. pylori* infection in gastric biopsy specimens (27). To our knowledge, our results obtained with the characterized isogenic *H. pylori* strains are the first to indicate that CagA does not influence induction of MIP-2.

Finally, a significant upregulation of the c-*myc* proto-oncogene by up to 6.8-fold (P17) was demonstrated upon infection with all *H. pylori* strains. These findings are consistent with those of Nardone et al., who showed enhanced prevalence of c-*myc* expression in patients with gastric atrophy who were chronically infected with type I strains (13).

Expression of FAK and LIMK is regulated in a CagA-dependent manner. Data from array analysis were confirmed by quantitative evaluation of Northern blots. FAK and LIMK are both involved in signaling cascades regulating organization of the actin cytoskeleton (28, 29). As a consequence, FAK and LIMK are both involved in regulation of cell motility and growth control. LIMK overexpression appears to retard cell growth indirectly by affecting processes related to cell proliferation, such as cytoskeletal organization (9), whereas overexpression of FAK leads to increased cell migration and survival (11).

Northern blots revealed a very low basal transcription of LIMK in AGS cells which was increased by up to 3.5-fold (G27) upon incubation with *H. pylori* wild-type strains. Expression of LIMK mRNA was 2.5-fold lower in P17-treated AGS cells compared to cells infected with the wild-type P12. Similar results were also achieved with the other pairs of isogenic strains differing in *cagA* presence or absence (Table 3). In contrast, transcription of FAK was decreased to 4.8-fold after exposure to *H. pylori* wild-type strains P12 and G27. Furthermore, incubation with a *cagA*-negative strain led to upregulation of FAK mRNA by up to 3.5-fold in AGS cells compared to infection with the wild type. With respect to both kinases, results similar to those shown with *cagA*-negative mutants were obtained by infection with strain 10-B4 (*cagE*-negative) (Table 3). Hence, it can be assumed that translocation of CagA

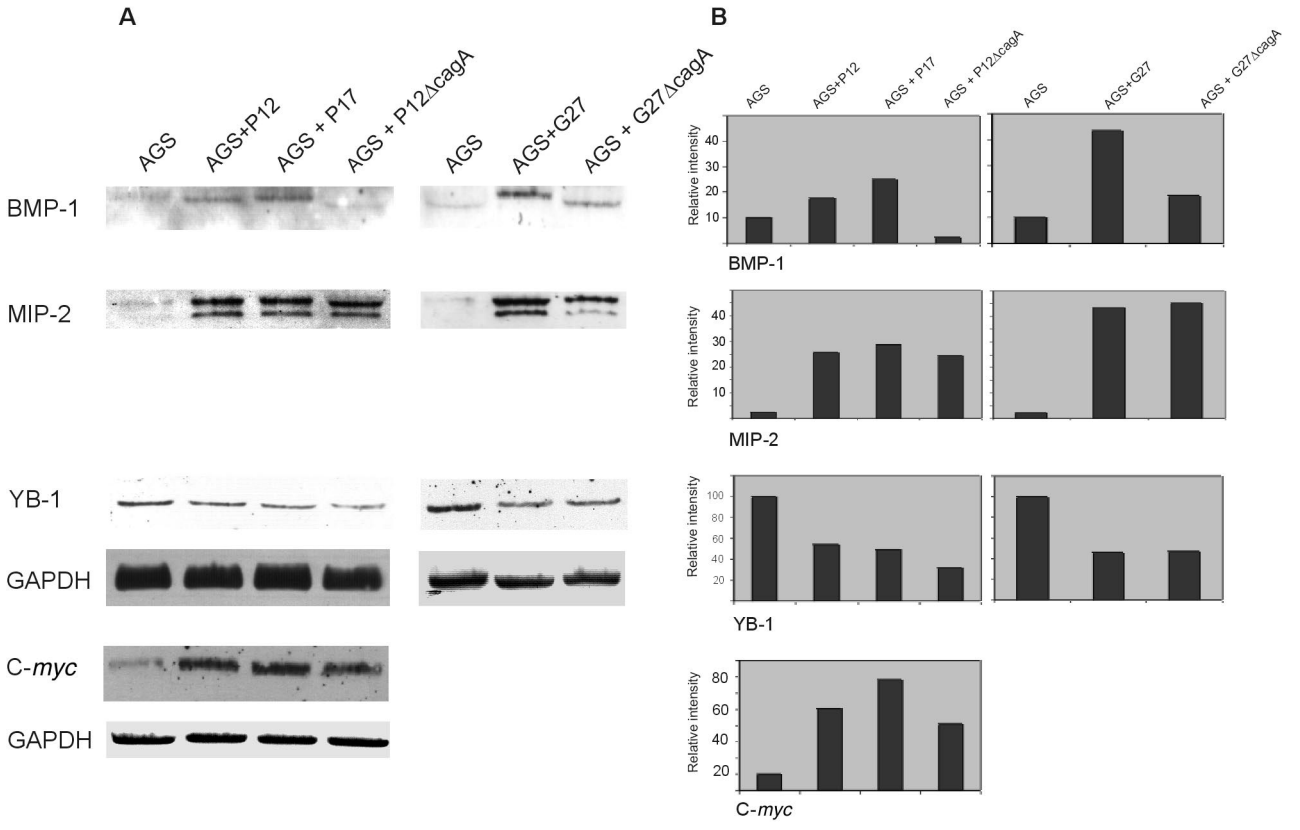

FIG. 2. Representative series of Northern blots hybridized to the indicated gene-specific probes. Three independent experiments were performed with similar results. After 4.5 h of coincubation using either wild-type P12 and *cagA*-negative isogenic strain P17 or P12Δ*cagA* or the isogenic pair G27 and G27Δ*cagA,* total RNA was isolated and subjected to Northern blotting as described (A). Total RNA from uninfected AGS cells was included as a control. The blots were hybridized to one probe, stripped, and rehybridized sequentially with the other probes indicated. Quantitation is indicated as fold intensity versus the control (B).

through the putative type IV secretion apparatus might be necessary for transcription modulation of both genes.

A role for CagA has been proposed for development of a specific cellular phenotype leading to dramatic elongation and spreading of gastric epithelial cells, which superimposes the stress fiber-associated (SFA) morphology induced by *H. pylori* attachment (22). Signal transduction pathways induced by attachment of *H. pylori* type I causing the morphological changes are not well characterized. Recently, involvement of small Rac GTPase in the signal transduction cascade leading to actin reorganization induced by *H. pylori* has been shown (18). Rho and Rac have both been shown to induce activation of LIMK, suggesting that these two G-proteins are mutually integrated in a highly dynamic process to regulate LIMK action and actin reorganization (17, 28).

FAK is a peripheral membrane protein mainly located in focal adhesions, where active stress fibers contact the cell membrane. A role of FAK in integrin-mediated activation of Rac and actin cytoskeleton organization has been described (4). In this context, it should be considered that FAK may play a role in SFA phenotype, which is inevitably caused by all *H. pylori* isolates. Downregulation of FAK and upregulation of LIMK might be regarded as an indication of the involvement of CagA in downregulating the SFA cellular phenotype in infected cells to favor development of the specific morphological phenotype caused by type I isolates.

Following CagA translocation, dephosphorylation of host cell proteins in the size range of 80 kDa and 120 to 130 kDa is observed (15). Interestingly, FAK has a molecular mass of 125

TABLE 3. Decrease or increase in LIMK and FAK expression due to incubation of AGS cells with pairs of isogenic *H. pylori* strains for 4.5 h[a]

Cells + strain	FAK expression (fold)		LIMK expression (fold)	
	Decrease versus uninfected control	Increase versus wild type	Increase versus uninfected control	Decrease versus wild type
AGS + P12	4.78 ± 0.07*		2.98 ± 0.13*	
AGS + P17	1.28 ± 0.17	3.01 ± 0.17*	1.17 ± 0.19	2.54 ± 0.19*
AGS + P12Δ*cagA*	1.27 ± 0.18	3.48 ± 0.18*	1.46 ± 0.16	2.04 ± 0.16*
AGS + G27	4.54 ± 0.10*		3.49 ± 0.52*	
AGS + G27Δ*cagA*	1.43 ± 0.08	3.19 ± 0.08*	1.38 ± 0.17	2.52 ± 0.17*
AGS + 10-B4 (*cagE*)	1.31 ± 0.11	3.47 ± 0.11*	1.25 ± 0.15	2.79 ± 0.15*

[a] Data from three independent Northern blot experiments were quantitatively evaluated, and means were compared statistically. *, $P < 0.01$ versus indicated control.

kDa. The *Yersinia* YopH protein, which is a highly active tyrosine phosphatase and disrupts focal adhesions by dephosphorylating FAK (19), is also delivered into the eukaryotic cell by a type III secretion system functionally similar to type IV and effectively inhibits bacterial uptake by epithelial cells and macrophages. It may therefore be hypothesized that CagA or other products of the *cag* PAI may play a similar role by regulating FAK activity. However, only divergent data on *H. pylori* resistance to phagocytosis are available at present (2, 15, 20).

In summary, differential expression of FAK and LIMK was clearly confirmed to be influenced by the presence of CagA in this study. However, from our recent data, we cannot definitely conclude whether the activities of LIMK and FAK are affected by CagA. Further functional studies to investigate if and how the biological activities of FAK and LIMK are influenced as a consequence of infection with *H. pylori* type I strains are currently being performed.

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