INFECTION AND IMMUNITY, June 2004, p. 3646–3649 Vol. 72, No. 6
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.6.3646–3649.2004

Helicobacter pylori Induces AGS Cell Motility and Elongation via Independent Signaling Pathways

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Received 15 December 2003/Returned for modification 12 January 2004/Accepted 2 March 2004

Helicobacter pylori **induces motogenic and cytoskeletal responses in gastric epithelial cells. We demonstrate that these responses can be induced via independent signaling pathways that often occur in parallel. The *cag* pathogenicity island appears to be nonessential for induction of motility, whereas the elongation phenotype depends on translocation and phosphorylation of CagA.**

Helicobacter pylori has been identified as the major etiological agent in the development of chronic gastritis and duodenal ulcer, and it plays a role in the development of gastric carcinoma. Virulent *H. pylori* strains harbor a pathogenicity island (*cag* PAI) for delivery of the bacterial CagA protein into AGS gastric epithelial cells (1–6, 8–12). After translocation, CagA is phosphorylated by Src family kinases at the tyrosine residue in the EPIYA sequence repeats (10–12). Phosphorylation of CagA is accompanied by high motility and elongation of the cells, the so-called hummingbird phenotype (2–4, 7–13). CagA has been shown to interact with a number of host signaling molecules, such as (i) the tyrosine phosphatase SHP-2 (7), (ii) C-terminal Src kinase (Csk) (13), (iii) Src itself, to induce inactivation of Src kinase and dephosphorylation of cortactin (11), (iv) c-Met receptor or phospholipase C-γ (4), (v) ZO-1 (1), and (vi) adaptor molecule Grb-2 (8).

In many of the above-mentioned studies, induction of the hummingbird phenotype has been considered a single *cag* PAI- and CagA-dependent process, although the contribution of different binding factors and signaling pathways for this phenotype remains controversial. To test whether the induced motility and cellular elongation of AGS cells are triggered by the same signaling pathway, we used live cell imaging to monitor and quantitate the effect of wild-type *H. pylori* and the corresponding isogenic *cag* PAI mutants on the motility and elongation of AGS cells. For this purpose, AGS cells (ATCC CRL 1739) were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS) in glass-bottom petri dishes (3.5 cm; Mattek Corp, Ashland, Mass.) to reach monolayers of approximately 50% cell confluence. The AGS monolayers were serum starved for 12 h and then infected at a multiplicity of infection of 100 with *H. pylori* strain P12, which carries a functional *cag* PAI (2). All infections were synchronized by centrifugation for 5 min at 600 × *g*, and the infection process of individual AGS cell colonies was recorded over 4 h by using a Zeiss Axiovert 200 M inverted microscope (Jena, Germany). We observed two distinct stages of behavior of the infected cells. During the first phase (60 to 90 min postinfection), a pronounced motogenic response was observed (Fig. 1A). The migratory cells exhibited no signs of cell elongation. In contrast, during the second phase (120 to 240 min postinfection), we observed that the migrating cells started to elongate and adopt a spindle-like morphology (Fig. 1A). Thus, there are apparently two distinct host responses, namely, motility and elongation, which may be induced sequentially by *H. pylori* during the early stage of infection. It is noteworthy that while the cells showed a sustained migratory activity throughout infection, the appearance of spindles is a dynamic process. For example, cell number 4 shows elongation between 120 and 240 min of infection, and cell number 3 shows temporal elongation at the 120-min time point (Fig. 1A).

To examine the dynamic migratory response and actin cytoskeletal rearrangements in more detail, AGS cells were transfected with an actin-enhanced green fluorescent protein construct (BD Biosciences Clontech, Heidelberg, Germany) for 16 h by using Lipofectamine 2000 transfection reagent (Invitrogen, Karlsruhe, Germany) followed by infection with wild-type *H. pylori*. The resulting phenotypical changes were monitored by time-lapse microscopy (Fig. 1B). A single AGS cell that started to exhibit strong motility between 25 and 100 min is indicated in Fig. 1B. Small elongations were sporadically observed between 125 and 175 min. Then, the cell moved in another direction without a significant sign of elongation (200 to 375 min). Finally, it formed very long elongations accompanied by drastic actin cytoskeletal rearrangements 450 to 650 min postinfection. This demonstrates that motility and elongation are dynamic processes that occur throughout infection.

Next, we investigated the importance of the *cag* PAI for phenotypical outcome. To ensure a high degree of fitness of both wild-type and mutant *H. pylori*, bacteria were grown for 12 h at 37°C with agitation at 200 rpm in 10 ml of liquid brain heart infusion medium (Difco Laboratories, Sparks, Md.) until they reached an optical density at 600 nm of 0.6. The infection was followed over 4 h by live cell imaging as described above. Migration of AGS cells was considered to have occurred when the cells in a certain cluster had lost their cell-to-cell contact and moved by a distance of at least 30 μm (Fig. 1 and 2). We found that wild-type *H. pylori* was able to induce a motogenic response in about 45% of the infected AGS cells (Fig. 2B and

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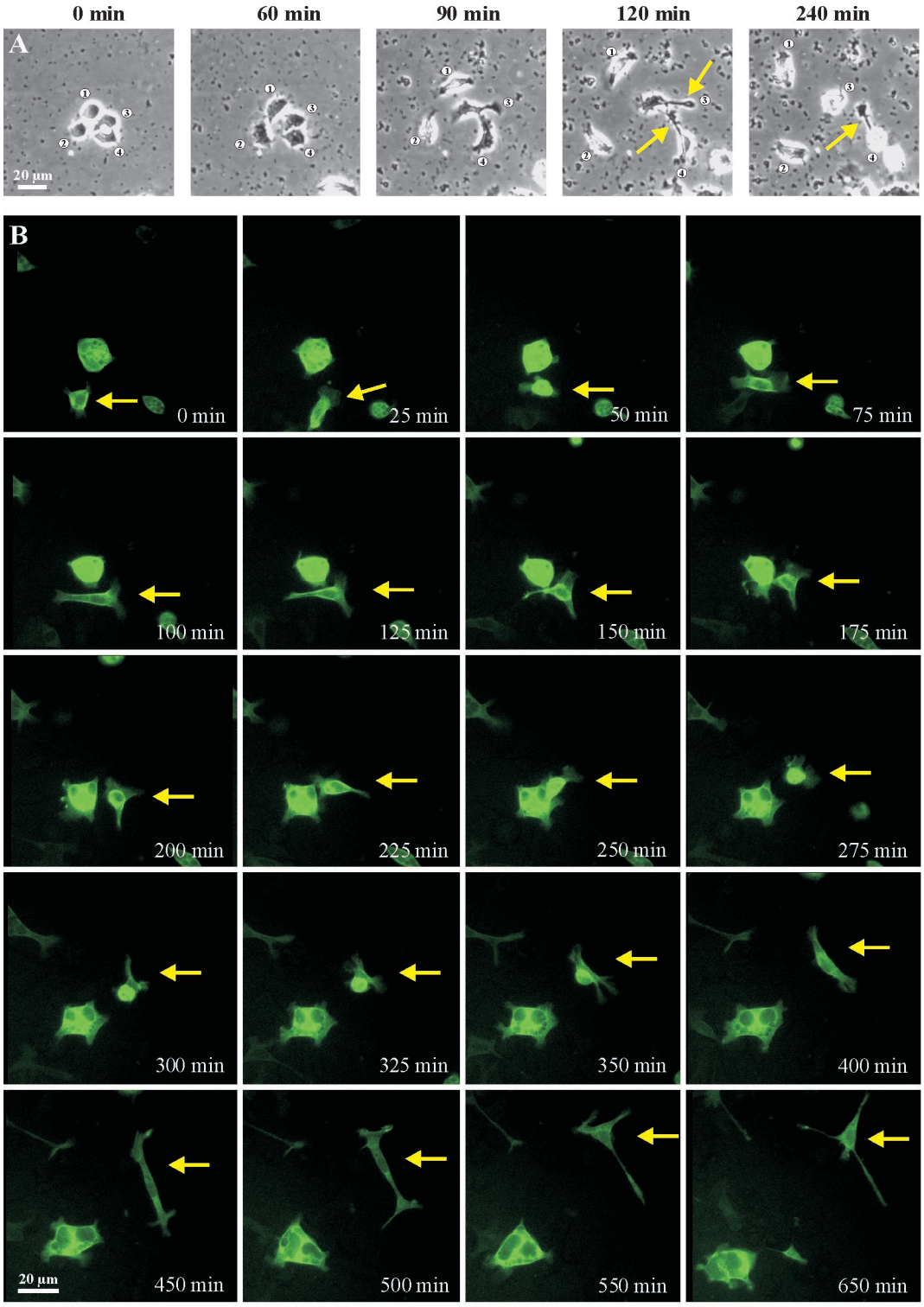

FIG. 1. Infection of AGS cells with *H. pylori* results in two early cellular phenotypes: the stimulation of migratory behavior and dramatic elongation of the host cells. (A) Single AGS cell colonies were investigated in a time course during infection with wild-type strain P12 by time-lapse phase-contrast microscopy as indicated. Cells shown are numbered 1 to 4. Arrows indicate those cells with significant elongation. (B) Investigation of the migratory behavior and dramatic elongation of a single AGS cell infected with *H. pylori* strain P1. The AGS cell marked by the arrows expresses a transfected actin-enhanced green fluorescent protein construct to monitor actin cytoskeletal rearrangements over 650 min of infection.

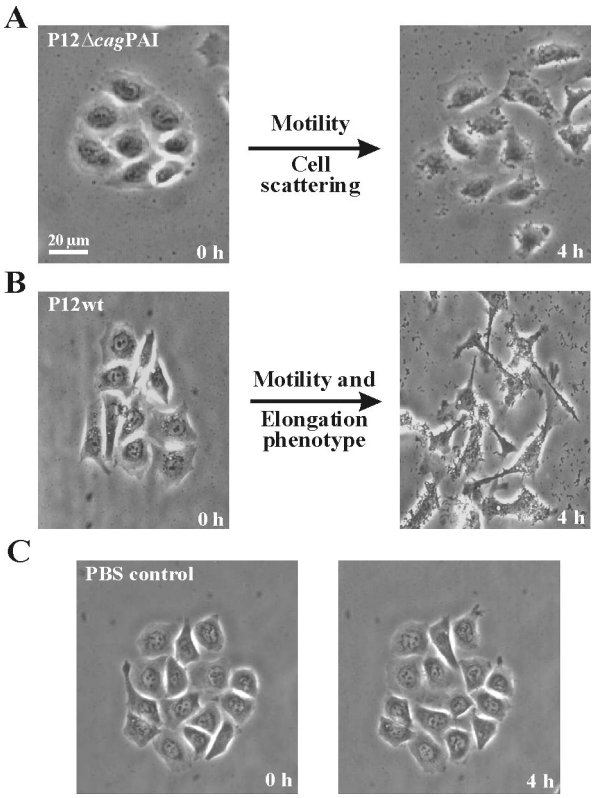

FIG. 2. Stimulation of migratory behavior and dramatic elongation of AGS cells with *H. pylori* strain P12Δ*cag*PAI (A) and wild-type (wt) strain P12 (B). Single AGS cell colonies that were investigated in a time course by time-lapse phase-contrast microscopy are indicated. The *cag* PAI appears to be nonessential for induction of motility, whereas the elongation phenotype requires a functional *cag* PAI. (C) Noninfected AGS control cells showed none of the responses. PBS, phosphate-buffered saline.

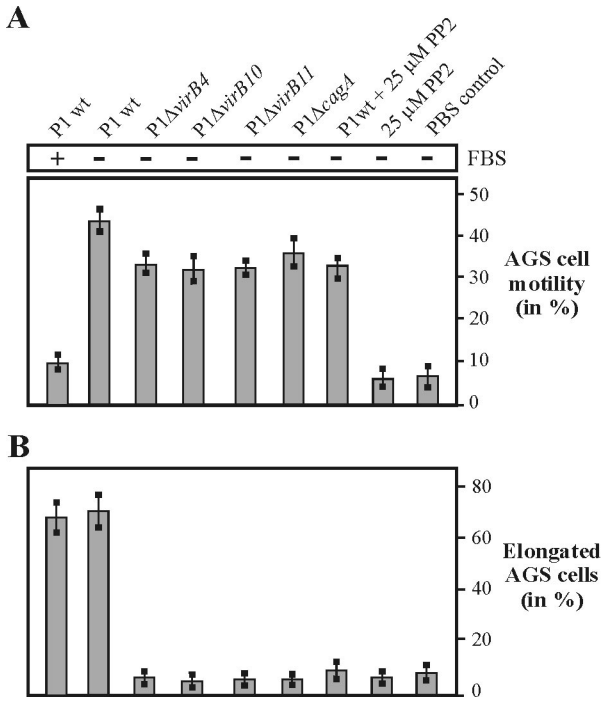

FIG. 3. Quantification of the motility (A) and elongation (B) phenotypes during infection of AGS cells with *H. pylori* wild type (wt) and *cag* PAI mutants. The infections were carried out with or without FBS as indicated. The Src kinase inhibitor PP2 was added 30 min prior to infection as indicated. A particular field of the coverslip was labeled and photographed before infection and at 4 h postinfection. One hundred cells were counted and evaluated from each photograph. The results are the means of three independent experiments. PBS, phosphate-buffered saline.

3A; Table 1). Surprisingly, isogenic mutants lacking either individual *cag* PAI genes or the entire *cag* PAI were still able to induce a substantial stimulatory effect in more than 30% of the infected AGS cells (Fig. 2A and 3A; Table 1). Interestingly, similar results were obtained with either the *cagA* deletion mutant or *cagA* mutants carrying phenylalanine substitutions in the proposed tyrosine phosphorylation sites (Y122F, Y899F, or Y918F) and the major phosphorylation site (Y972F), which showed only slightly reduced motility rates (ca. 35 to 40%) compared to that of wild-type bacteria (Fig. 3A; Table 1). Thus, although we cannot discount at this stage a minor contribution of the *cag* PAI or translocated CagA itself to host cell signaling leading to a migratory response, the results strongly suggest that the major determinant for the induction of AGS cell motility is likely to be encoded outside the *cag* PAI.

Finally, we quantified the elongation phenotype of AGS cells upon infection with the same set of strains by counting the number of AGS cells which show thin, needle-like protrusions of 20 to 70 μm in length (2, 9). Smaller protrusions (<10 μm) that were also occasionally seen in the uninfected control cells were not counted. The results obtained (Fig. 2A and B and 3B; Table 1) show that in contrast to the *cag* PAI-independent induction of cellular motility, the elongation phenotype of infected AGS cells clearly depends on functional *cag* PAI genes as well as translocation and phosphorylation of CagA on tyrosine residue 972.

Further evidence for motility and elongation being two independent host cell responses came from two other observations. First, when the AGS cells were infected with *H. pylori* in the presence of FBS, we observed a drastic reduction in cellular motility (Fig. 3A) with minimal effect on elongation (Fig. 3B). This argues strongly that induction of a marked motility is not essential for the elongation phenotype. Second, the presence of pharmacological inhibitor PP2 (Merck, Schwalbach, Germany) at concentrations specific for Src family kinases (10, 12) blocked the elongation phenotype by inhibiting CagA phosphorylation on tyrosine 972 (Fig. 3B) (2), whereas it did not significantly affect the induction of motility (Fig. 3A; Table 1). Thus, different signaling seems to be essential for induction of cellular motility and elongation, respectively.

Taken together, our findings indicate that the induction of AGS motility and elongation are two independent events which often occur in parallel or shortly one after the other and are likely to be triggered by different signaling pathways. This conclusion is further supported by a recent study from our labs which reports on the characterization of clinical *H. pylori* isolates from 75 patients (3). Interestingly, we found three types of clinical isolates inducing the following phenotypes: (i) motility positive, elongation positive; (ii) motility positive, elongation negative; and (iii) motility negative, elongation negative. These findings strongly support the notion that the induction of motility and the induction of cellular elongation are two processes which can be triggered by different signaling

TABLE 1. Induction of AGS motility and elongation in infections with *H. pylori cag* PAI mutants[a]

H. pylori strain	Mutated ORF	Gene name	Induction of AGS cell		Translocation and phosphorylation of CagA[d]
			Motility[b]	Elongation[c]	
P1 wt			+++	+++	+++
P1Δ*cag3*	522	*cagD*	++	−	−
P1Δ*cag5*	524	*virD4*	+++	−	−
P1Δ*cag*α	525	*virB11*	++	−	−
P1Δ*cag7*	527	*virB10*	++	−	−
P1Δ*cag8*	528	*virB9*	++	−	−
P1Δ*cag9*	529	*virB8*	++	−	−
P1Δ*cag12*	532	*virB7*	++	−	−
P1Δ*cag13*	534	*cagS*	++	−	−
P1Δ*cag16*	537	*cagM*	++	−	−
P1Δ*cag17*	538	*cagN*	++	−	−
P1Δ*cag19*	540	*cagI*	++	−	−
P1Δ*cag23*	544	*virB4*	++	−	−
P1Δ*cag26*	547	*cagA*	+++	−	−
P1Δ*cag26*	547	*cagA* Y122F	+++	+++	+++
P1Δ*cag26*	547	*cagA* Y899F	+++	+++	+++
P1Δ*cag26*	547	*cagA* Y918F	+++	+++	+++
P1Δ*cag26*	547	*cagA* Y972F	+++	−	−
P12 wt			+++	+++	+++
P12Δ*cag*PAI	519–548	*cag* PAI	++	−	−
B128 wt			+++	+++	+++
B128Δ*cag*PAI	519–548	*cag* PAI	++	−	−

[a] AGS cells were infected with the indicated *H. pylori* strains at a multiplicity of infection of 100 for 4 h. Cellular motility and elongation were measured as described in the text. Nomenclature of the mutated genes is according to TIGR *H. pylori* strain 26695 (http://www.tigr.org) and Covacci and Rappuoli (5). *H. pylori* strain P1 and its isogenic mutants have been described elsewhere (2, 11). Mutagenesis and complementation of *cagA* expressed from a shuttle vector (*cagA* Y122F, Y899F, Y918F, and Y972F) were reported previously (2). Strains P12 and B128 (and their respective *cag* PAI deletion mutants) were gifts of Rainer Haas (Max von Pettenkofer Institute, Munich, Germany) and Richard M. Peek, Jr. (Vanderbilt University, Nashville, Tenn.), respectively. wt, wild type; ORF, open reading frame.

[b] +++, high inducers (40 to 50% motility); ++, moderate inducers (30 to 40% motility). Phosphate-buffered saline control levels of AGS cell motility were around 5 to 7%.

[c] +++, high inducers (70 to 85% elongated cells); −, control level. Phosphate-buffered saline control levels of AGS cell elongation were around 5 to 8%. No intermediate levels of elongation were observed.

[d] Translocation and phosphorylation of CagA were determined by standard Western blotting procedures using anti-CagA and antiphosphotyrosine antibodies (2, 11). +++, strong phosphorylation; −, no phosphorylation.

Editor: D. L. Burns

pathways and bacterial factors. Our findings set the stage for a more precise definition of the hummingbird phenomenon with respect to cellular motility and cell elongation, a necessary basis for the better understanding of CagA signaling and the molecular mechanism involved in these cell responses.

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