A Transgenic Drosophila Model Demonstrates That the Helicobacter pylori CagA Protein Functions as a Eukaryotic Gab Adaptor

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Abstract

Infection with the human gastric pathogen Helicobacter pylori is associated with a spectrum of diseases including gastritis, peptic ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma. The cytotoxin-associated gene A (CagA) protein of H. pylori, which is translocated into host cells via a type IV secretion system, is a major risk factor for disease development. Experiments in gastric tissue culture cells have shown that once translocated, CagA activates the phosphatase SHP-2, which is a component of receptor tyrosine kinase (RTK) pathways whose over-activation is associated with cancer formation. Based on CagA’s ability to activate SHP-2, it has been proposed that CagA functions as a pro-kinolytic mimic of the eukaryotic Grb2-associated binder (Gab) adaptor protein, which normally activates SHP-2. We have developed a transgenic Drosophila model to test this hypothesis by investigating whether CagA can function in a well-characterized Gab-dependent process: the specification of photoreceptors cells in the Drosophila eye. We demonstrate that CagA expression is sufficient to rescue photoreceptor development in the absence of the Drosophila Gab homologue, Daughter of Sevenless (DOS). Furthermore, CagA’s ability to promote photoreceptor development requires the SHP-2 phosphatase Corkscrew (CSW). These results provide the first demonstration that CagA functions as a Gab protein within the tissue of an organism and provide insight into CagA’s oncogenic potential. Since many translocated bacterial proteins target highly conserved eukaryotic cellular processes, such as the RTK signaling pathway, the transgenic Drosophila model should be of general use for testing the in vivo function of bacterial effector proteins and for identifying the host genes through which they function.

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Introduction

The human pathogen, Helicobacter pylori, infects the stomachs of at least half the world’s population and chronic infection is associated with the development of diseases such as gastritis, peptic ulcers and gastric cancer [1]. A major virulence determinant of H. pylori is the cytotoxin associated gene A (CagA) which is translocated into host cells via a type four secretion system [reviewed in [2]]. Inside host cells, CagA is phosphorylated by Src family kinases on tyrosines contained in repeated five-amino acid motifs (EPIYA) in CagA’s carboxyl terminus. Phosphorylated CagA disrupts receptor tyrosine kinase (RTK) signaling pathways by directly activating Src homology 2 (SH2) domain containing tyrosine phosphatase (SHP-2) [reviewed in [3]]. Normally SHP-2 is activated by the scaffolding adaptor Grb2-associated binder (Gab) proteins, thereby amplifying RTK signaling pathways to control cell growth, differentiation and survival [reviewed in [4]]. The Gab proteins occupy a pivotal position in RTK signaling pathways by interacting directly with RTKs such as the c-Met receptor of the Hepatocyte growth factor/Scaetter factor (HGF/SF) as well as downstream cytoplasmic proteins including SHP-2, v-crk sarcoma virus CT10 oncogene homolog (avian)-like (Crk[L]), and Growth factor receptor-bound protein 2 (Grb2) [reviewed in [5,6,7]]. Although CagA shares no sequence similarity with Gab proteins, CagA has been shown to activate SHP-2 in tissue culture cells, resulting in cell elongation [8,9]. Similarly, in tissue culture cells CagA has been found to associate with c-Met, Crk(L) and Grb2 [10,11,12]. Based on these interactions, CagA has been hypothesized to mimic Gab proteins and to function as an oncogene by over-activating RTK signaling [13]. The significance of CagA’s interactions with RTK signaling pathway proteins, however, has only been explored in tissue culture cells.

We have developed transgenic Drosophila with inducible CagA expression as a model to understand CagA’s mechanisms of action in complex epithelial tissues. In order to test the hypothesis that CagA can function as a Gab substitute, we investigated CagA activity in a well-characterized Gab-dependent process, the specification of photoreceptors in the Drosophila eye [14,15,16]. The Drosophila compound eye, whose crystalline array of facets or ommatidia are exquisitely sensitive to perturbations in cell specification, has been used as a powerful system for the discovery and genetic analysis of RTK signaling components [17,18].
Drosophila RTK signaling proteins are highly conserved with their mammalian orthologues and oncogenic mutations in these proteins, such as those that constitutively activate RTK receptors or their downstream effectors, function similarly in both Drosophila and mammalian cells [19]. The Drosophila model also offers elegant tools for genetic manipulations including the UAS/GAL4 system [20] for expression of transgenes in a tissue specific manner, the FLP/FRT system for the generation of somatic mutant clones [21], and null mutations in most RTK signaling pathway members, which allow us to probe the in vivo requirements for CagA's activation of RTK signaling pathways. Finally, Drosophila are amenable to forward genetic approaches that will facilitate the discovery of host factors required for CagA function in eukaryotic cells [22].

RTK signaling is required for multiple steps of Drosophila photoreceptor development. The Drosophila epidermal growth factor receptor (EGFR) is necessary for cell proliferation in the early eye imaginal disc, cell survival in the differentiating region of the disc behind the morphogenetic furrow, and recruitment of all photoreceptors except R8 [23]. A second RTK, Sevenless (SEV) is required exclusively for the R7 photoreceptor to adopt the appropriate fate, as opposed to becoming a nonneuronal cone cell [24] (reviewed in [25]). The Drosophila Gab adaptor, Daughter of Sevenless (DOS) is required for full signaling through both the EGFR and SEV pathways [16]. Clones of eye imaginal cells lacking DOS activity fail to proliferate and produce few photoreceptors, similar to clones lacking EGFR [16,26,27]. The EGFR pathway is required additionally for multiple aspects of Drosophila development [28].

Here we show that CagA can substitute for the Drosophila Gab adaptor, DOS, and rescue phenotypes associated with loss of dos, including larval lethality and photoreceptor differentiation. We further demonstrate that CagA functions through the Drosophila SHP-2 homologue, Corkscrew (CSW) similar to Gab. Our work demonstrates the power of using a genetically tractable system like Drosophila to dissect the mechanism of action of a prokaryotic protein that modulates a conserved eukaryotic signaling pathway.
pupae that should develop if the dos mutants showed no lethality defect. As expected, a low percentage (33%) of homozygous dos mutant pupae expressing only Hsp-GAL4 were observed (Figure 2A). When CagA was expressed, we observed a significant increase to 89% of the pupae developing that lacked dos (Figure 2A). These results indicate that CagA can substitute for essential functions of DOS during Drosophila development.

To specifically test whether CagA could substitute for Gab in photoreceptor development, we generated mitotic dos/dos clones within the eye using the FLP/FRT recombinase system [27,32]. In these experiments the dos mutation was recombined onto a chromosome arm containing a centromere proximal FRT recombination site and maintained in trans to a chromosome containing the same FRT site as well as a GFP transgene. By expressing FLP recombinase in the developing eye we induced mitotic recombination between FRT sites, which generated clones of homozygous cells (dos/dos) in an otherwise heterozygous background (dos/+). The dos/dos mutant cells were distinguished by their lack of GFP, and the photoreceptors were visualized by staining for the photoreceptor-specific protein

Figure 1. CagA is phosphorylated, associates with the cortex in Drosophila cells and disrupts eye development. (A) UAS-CagA was expressed in the Drosophila eye with GMR-GAL4. CagA protein (α-HA) was tyrosine phosphorylated (α-P-Tyr). Controls expressed only GMR-GAL4. (B) The CagA and CagAEPISA proteins were expressed in the eye to similar levels. (C) CagA (α-HA, green) localized to the cortex (phalloidin, red) of cells in the larval eye disc. Wild type (D) and UAS-CagA/GMR-GAL4 (E) pupal retinas were stained with MAb 24B10 (red) to outline the photoreceptors, and antibodies to visualize the R7 (green) and R8 (cyan) photoreceptors. Scanning electron microscope micrographs of adult eyes from flies with (F) one copy of GMR-GAL4 and no UAS transgene, (G) one copy of UAS-CagA, (H) two copies of UAS-CagA and (I) one copy of UAS-CagAEPISA. doi:10.1371/journal.ppat.1000064.g001
As previously reported [16,26] the dos/dos clones rarely contained photoreceptors and were composed of very few cells (Figure 2B–E), due to the dual requirements for EGFR signaling in cell survival and photoreceptor specification [23]. As expected, expression of DOS with GMR-GAL4 in dos/dos cells resulted in much larger clones with increased numbers of photoreceptors (Figure 2B, F–H). Expression of CagA in dos/dos cells was able to rescue clone size and photoreceptor development similarly to expression of DOS with the same driver (Figure 2B, I–K). Two independent dos mutants gave similar results (Figure 2 and data not shown). These data demonstrate that CagA can substitute for DOS during the development of photoreceptors.

CagA's specification of photoreceptors requires SHP-2/CSW

We predicted that if CagA functions similarly to Gab, then CagA would require the downstream signaling molecule SHP-2/CSW to promote photoreceptor development. As a downstream component of RTK pathways, CSW is required for photoreceptor development.
CagA, like DOS, requires SHP-2/CSW to promote photoreceptor mutants (Figure 3D, E, data not shown). These results argue that CagA, like DOS, increases the number of photoreceptors in two different mutants with or without CagA expression. (A) Wild type larval eye discs contain thousands of photoreceptors, which were visualized by anti-ELAV staining. (B) Few photoreceptors develop in csw null mutant larval eye discs. (C) Expression of UAS-CSW using GMR-GAL4 in the csw mutant partially rescued the lack of photoreceptor development but expression of UAS-CagA did not (D). (E) There was no significant difference (T-test, p value >0.2) in the number of photoreceptors (PR) that developed in csw mutants with or without CagA expression.

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Discussion

We used a transgenic Drosophila system to test the hypothesis that H. pylori's virulence factor CagA can substitute for the Gab adaptor in RTK signaling pathways. This system is ideal for these studies because RTK signaling pathway components can be genetically manipulated, resulting in interpretable phenotypic consequences for tissue development. First, we have demonstrated that CagA in Drosophila tissue is phosphorylated, that it associates with the cell cortex, and that its expression causes epithelial disorganization as in mammalian tissue culture cells. Second, we have provided genetic evidence that CagA can substitute for Gab by demonstrating that CagA expression restores larval viability and photoreceptor development in mutants lacking the Drosophila Gab, DOS. Our inability to rescue dos mutants to adulthood with CagA expression may be due to differences in RTK activation or to non-overlapping functions of Gab and CagA. Indeed too much CagA expression (using an actin-GAL4 driver) is lethal to flies (unpublished results), which is not the case for ubiquitous expression of DOS [26]. Third, our genetic epistasis analysis with mutants lacking csw has shown that CagA functions through the Drosophila SHP-2 homologue, similar to results from tissue culture experiments [8,9].

RTK signaling is essential for several fundamental biological processes and erroneous signaling can promote tumor formation [19]. Gain-of-function mutations of SHP-2 have been established as oncogenic in numerous leukemia types as well as other diseases like Noonan’s Syndrome [4,34,35]. Over-expression of the Gab scaffolding adaptor proteins is associated with the development of several types of cancers, including breast cancer [6,7] and gastric cancer [36]. The specific cancers that develop as a result of these mutations reflect tissue sensitivities to increased Gab and SHP-2. In the case of H. pylori infection, CagA provides a tissue specific activation of RTK signaling that can precipitate events leading to gastric carcinogenesis [37], as suggested by a recent report of CagA-expressing transgenic mice [38].

Our approach of examining the cellular effects of CagA expression in Drosophila tissue takes advantage of the fact that bacterial proteins frequently target essential, highly conserved cell-signaling pathways. Drosophila has been employed traditionally as a model organism for dissecting signaling pathways in development, but in recent years it has also proven useful in understanding host-pathogen interactions (reviewed in [39,40]), and in one instance has been used as a heterologous system for expression of the bacterial toxins, anthrax lethal and edema factors [41]. Here we have exploited Drosophila eye development to demonstrate CagA's capacity to function as a RTK adaptor. Future studies using this transgenic Drosophila model will allow us to better understand the cellular and tissue-wide consequences of CagA's disruption of eukaryotic signaling pathways and to identify candidate host factors through which CagA functions.

Materials and Methods

Construction of UAS-CagA and UAS-CagA\textsuperscript{EPISA}

CagA cDNA was amplified from genomic DNA from H. pylori G27. The CagA\textsuperscript{EPISA} (lacking EPIYA tyrosine phosphorylation motifs) cDNA was amplified from a plasmid provided by Manuel Amieva (originally from Markus Stein [42]). CagA\textsuperscript{EPISA} lacks the tyrosines in the four 5-amino acid motif, EPIYA, which are phosphorylated by host kinases (point mutations at nucleotide 2684 [A→C] and 2740 [A→C] and a deletion at nucleotide 2878 to 3082). CagA and CagA\textsuperscript{EPISA} were cloned into a modified pUAST vector with an N-terminal hemagglutinin (HA) tag (provided by...
Chris Q, Doe). Transgenic lines were generated by injecting Qiagen-purified plasmid DNA into y,w,118 embryos. Several independent transformant lines were established for each construct.

**Drosophila Strains**

Genetic null alleles of csw (csw^{G114} and csw^{15-87}) and dos (dos^{1.46} and dos^{7.69}) were obtained from Michael Simon. The UAS-DOS strain was from Thomas Raabe and the UAS-CSW strain (UAS-

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**Scanning Electron Microscopy**

Fly heads were fixed overnight at 4°C in 2% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated through an ethanol series (30%, 50%, 70%, 80%, 90%, 95%), three times in absolute ethanol) at room temperature for 10 minutes in each solution. Samples were critically point dried, sputter coated with gold and viewed using a JEOL 6400 SEM.

**Pupal Retinas**

Wandering third instar larvae were placed at 25°C and approximately 50 hours later the pupal retinas were dissected (50% pupal stage). Retinas were dissected in PBS, fixed for 20 minutes (4% paraformaldehyde in PBS) and washed three times in PBT (0.5% Triton X-100 in PBS). Retinas were blocked at least 15 minutes in 10% normal goat serum in PBT. Antibodies were diluted in the blocking solution. Primary antibodies included mouse MAb 24B10 which stains all photoreceptors and their axons [43] and goat anti-GFP antibodies. The heads from the bodies using a mesh sieve. Heads (~1.5 mL) were homogenized in ice cold lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 0.5% Triton X-100 and Complete protease inhibitors [Roche]) and then centrifuged at 16,000 G for 5 minutes. Supernatant from the lysate solution (1.5 mL) was added to 50 μL anti-HA Affinity Matrix (Roche) which was incubated overnight at 4°C with gentle agitation. The anti-HA affinity matrix was washed 4 times with ice-cold lysis buffer. CagA was eluted from the matrix by boiling in 100 uL sample loading buffer and separated using manufacturers protocols for 7% NuPAGE® Novex Tris-Acetate gels, transferred to polyvinylidene difluoride membranes, blocked overnight at 4°C (200 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20 and 3% BSA (Fisher), probed using appropriate antibodies and detected using enhanced chemiluminescence (ECL, plus, Amersham Biosciences), Mouse anti-HA was used at 1:1,000 (Babco). Mouse anti-phospho tyrosine was used at 1:2,000 (Cell Signal Technologies).

**Western Analysis**

Fly heads were collected by flash freezing adult flies in liquid nitrogen, shaking the flies in a conical tube, and then separating the heads from the bodies using a mesh sieve. Heads (~1.5 mL) were homogenized in ice cold lysis buffer (50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 0.5% Triton X-100 and Complete protease inhibitors [Roche]) and then centrifuged at 16,000 G for 5 minutes. Supernatant from the lysate solution (1.5 mL) was added to 50 μL anti-HA Affinity Matrix (Roche) which was incubated overnight at 4°C with gentle agitation. The anti-HA affinity matrix was washed 4 times with ice-cold lysis buffer. CagA was eluted from the matrix by boiling in 100 uL sample loading buffer and separated using manufacturers protocols for 7% NuPAGE® Novex Tris-Acetate gels, transferred to polyvinylidene difluoride membranes, blocked overnight at 4°C (200 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween-20 and 3% BSA (Fisher), probed using appropriate antibodies and detected using enhanced chemiluminescence (ECL, plus, Amersham Biosciences), Mouse anti-HA was used at 1:1,000 (Babco). Mouse anti-phospho tyrosine was used at 1:2,000 (Cell Signal Technologies).

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Author Contributions
Conceived and designed the experiments: CB KG. Performed the experiments: CB AW. Analyzed the data: CB AW KG. Contributed reagents/materials/analysis tools: CB AW KG. Wrote the paper: CB KG.

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