Cell Vacuolation Induced by the VacA Cytotoxin of Helicobacter pylori Is Regulated by the Rac1 GTPase*

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Chronic gastric infection with the Gram-negative bacterium Helicobacter pylori is a major contributing factor in the development of duodenal ulcers and is believed to be a significant risk factor in the development of gastric tumors. The VacA cytotoxin of H. pylori is a 90-kDa secreted protein that forms trans-membrane ion channels. In epithelial cells, VacA activity is associated with the rapid formation of acidic vacuoles enriched for late endosomal and lysosomal markers. Rac1 is a member of the Rho family of small GTP-binding proteins that regulate reorganization of the actin cytoskeleton and intracellular signal transduction and are being shown increasingly to play a role in membrane trafficking events. In this study, we report that: (i) green fluorescent-tagged Rac1 localizes around the perimeter of the vacuoles induced by VacA; (ii) expression of dominant negative Rac1 in epithelial cells inhibits vacuole formation; (iii) expression of constitutively active Rac1 potentiates the activity of VacA. Taken together, these data demonstrate a role for Rac1 in the regulation of VacA activity.

Infection with the Gram-negative bacterium Helicobacter pylori is a major risk factor for development of duodenal ulcers and has been implicated in gastric cancer (1). One of the major determinants in Helicobacter disease is secretion of a 90-kDa toxin called VacA, which causes rapid intracellular vacuole formation in vitro and gastric ulceration in mice (2). These vacuoles are acidic and are believed to be a consequence of the reorganization of the late endosomal and lysosomal compartments (3). The associated increased release of acid hydrolases is believed to play a role in degradation of the protective mucus lining the stomach (4). Although it is accepted that VacA plays a key role in the pathogenesis of H. pylori, relatively little is known about how the toxin works or the intracellular mechanisms that regulate the reorganization of the late endosomal and lysosomal compartments. Recent data suggest that multiple VacA molecules may complex to form membrane-spanning pores, and there is evidence that implicates the GTP-binding protein Rab7 in the formation of the hybrid late endosomal-lysosomal compartments (5–8).

Rac1 is a member of the Rho family of small GTPases. Rac1 and other members of the Rho family are known to regulate the actin cytoskeleton and a number of intracellular signal transduction pathways (9). It is well established that over-expression of constitutively active mutants of Rac1 results in increased pinocytic activity, and more recent reports have indicated that Rac1 and other members of the Rho family play an important role in a variety of other membrane trafficking events (10–15). Members of the Rho family have also been implicated in the pathogenesis of a number of microorganisms including Listeria monocytogenes and Shigella flexneri (16, 17). Given the known links between Rac1, membrane trafficking, and microbial pathogenesis, we were interested in whether the pathogenic events associated with H. pylori infection are regulated by members of the Rho family. In this study, we demonstrate that activity of the VacA cytotoxin is regulated by Rac1.

EXPERIMENTAL PROCEDURES

Cell Culture—SCC12F keratinocytes were cultured using the method of Rheinwald and Green (18) as described elsewhere (19). MDCK cells, stably expressing N-terminal myc-tagged N17Rac1 and V12Rac1 under control of the tetracycline repressible trans-activator, were provided by James Nelson (Stanford University). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 20 ng/ml doxycycline as described elsewhere; expression of myc-tagged N17Rac1 and V12Rac1 was achieved by culturing cells in the absence of doxycycline for 16 h (20). Expression of V12Rac1 and N17Rac1 was monitored by indirect immunofluorescent staining using an antibody to the myc epitope (9E10). In all assays, ammonium chloride was added to a final concentration of 10 mM before the addition of VacA.

**Purification of VacA—**VacA was purified from H. pylori 60180 cultures, as described elsewhere (21). Before VacA was added to cell cultures, it was acidified by dropwise addition of 200 mM HCl to a final pH of 2.5.

**Construction of GFP-tagged Rac1 Constructs**—Construction and functional characterization of pCDNA3-GFP-pCDNA3-GFP-WT-Rac1 are described in detail elsewhere (22). Briefly, polymerase chain reaction was used to generate an open reading frame encoding GFP coupled to the N terminus of full-length wild type (WT) Rac1 via a -Gly-Gly-Gly-Ser linker that was subcloned into pCDNA-3.1(+)(Invitrogen). For control experiments a GFP expression vector was made by subcloning GFP into pCDNA-3.1(+). Plasmids were microinjected into the nuclei of SCC12F cells as described elsewhere (23).

**Neutral Red Uptake Assay—**MDCK cells were plated at low density in 96-well plates in the presence or absence of doxycycline and cultured for 16 h, after which VacA was added and cells left for a further 4 h. The vacuolating activity of VacA was analyzed using a previously published method, in which uptake of Neutral Red dye into acidic compartments is quantitated using a microtiter plate reader to determine absorbance at 540 nm (24). Background uptake of Neutral Red dye in the absence of VacA was subtracted from the values obtained in the presence of VacA to provide a measure of the vacuolating activity induced by VacA. Duplicate plates were analyzed using an image-based cell quantitation assay to correct for potential differences in cell numbers in each treatment (25). Triplicate wells were assayed for each condition and results expressed as the mean plus standard error from three separate experiments.

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1 The abbreviations used are: MDCK cells, Madin-Darby canine kidney cells; WT, wild type; GFP, green fluorescent protein.
RESULTS AND DISCUSSION

Rac1 is a member of the Rho family of small GTPases, known to regulate the actin cytoskeleton and a number of intracellular signaling events (9). Members of the Rho family, including Rac1, have been shown to be important for the pathogenic activity of a number of micro-organisms and are increasingly seen as important in membrane trafficking events (12, 13, 16, 17). We recently observed that expression of a constitutively active form of Rac1 in epithelial cells resulted in formation of multiple, large vesicular structures reminiscent of those seen in cells exposed to the VacA cytotoxin of H. pylori (22). VacA is a small pore-forming toxin, which acts by inhibiting endosomal biogenesis, resulting in the expansion of acidic compartments (2, 5). In this study, we have analyzed whether the vacuolating activity of VacA is regulated by Rac1.

Treatment of two different epithelial cell lines, SCC12F keratinocytes and MDCK cells, with 200 ng ml \(^{-1}\) VacA toxin for 4 h resulted in the formation of large intracellular vacuoles in some, although not all, cells (Fig. 1). Treatment of cells with VacA for longer periods (16–24 h) resulted in vacuolation of virtually all cells (data not shown). This vacuolation of non-gastric epithelial cells is consistent with the previously reported effects of this toxin on epithelial cells derived from a variety of different tissue sources (2, 26).

Expression of Rac1 in cells is associated with increased pinocytic activity and is believed to play a role in endocytosis (13, 27). Consistent with this role, Rac1 has been shown to localize around intracellular vesicles (22, 28). To analyze whether Rac1 localized to VacA-induced vesicles, we microinjected SCC12F cells with a cDNA vector expressing wild type Rac1 fused to green fluorescent protein (GFP) (22). Injected cells were incubated for 16 h to allow expression of the GFP-Rac1 chimera before the addition of VacA for a further 4 h. Expression of wild type GFP-Rac1 did not induce cell vacuole formation in the absence of VacA (Fig. 2, A and B), but in cells treated with VacA Rac1 it was clearly seen to localize to the periphery of the vacuoles (Fig. 2, C and D). Cells microinjected with a control plasmid expressing GFP alone and treated with VacA showed no specific localization of GFP around these vacuoles (Fig. 2, E and F).

Localization of Rac1 to VacA-induced vacuoles implicated Rac1 in the VacA-mediated response; we analyzed the role of Rac1 in this process using MDCK cells expressing dominant negative and constitutively active Rac1 mutants under the control of the tetracycline repressible trans-activator (20). Cells were cultured overnight in the absence of the tetracycline analogue doxycycline to induce expression of either dominant negative (N17) or constitutively active (V12) Rac1 mutants before adding VacA for a further 4 h. Expression of the Rac1 mutants was verified by staining cells for the presence of the myc epitope located at the N terminus of the mutants (20). No expression of dominant negative or constitutively active Rac1 was observed in the presence of doxycycline (data not shown). In the absence of doxycycline, dominant negative and constitutively active Rac1 was expressed in most, although not all, cells (Fig. 3, B, D, F, and H). In cells expressing constitutively active Rac1, the majority of cells demonstrated extensive vacuole formation in the presence of VacA (Fig. 3, C and D). In the absence of VacA, expression of constitutively active Rac1 did not result in vacuole formation (Fig. 3, A and B). In contrast to the results observed following expression of constitutively active Rac1, cells expressing dominant negative Rac1 showed markedly less vacuole formation (Fig. 3, E and F). As with the cells expressing constitutively active Rac1, expression of dominant negative Rac1 was variable with approximately 20–30% of cells not expressing N17Rac1 following withdrawal of doxycycline. Significantly, those cells in which dominant negative Rac1 was not expressed at detectable levels, as determined by indirect immunofluorescence, appeared more susceptible to the effects of VacA (Fig. 3, G and H).

To confirm the apparent effects of expressing dominant negative and constitutively active Rac1 on the ability of VacA to induce vacuole formation in MDCK cells, we used a quantitative assay for vacuole formation based on uptake of Neutral Red dye into acidic vacuoles (24). Data from these experiments clearly support the finding that Rac1 regulates the cellular response to VacA toxin. Expression of dominant negative Rac1 inhibits vacuole formation by approximately 50%, and expression of constitutively active Rac1 results in a 2–3-fold increase in vacuole formation over and above that seen in cells express-
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In the absence of doxycycline to induce expression of the Rac1 mutants. In tetracycline repressible trans-activator were cultured for 16 h in the active (E), Rac1 under the control of the myc 4h. In the pathogenesis of a number of other micro-organisms, including endogenous Rac1 (Fig. 4). The inability of dominant negative Rac1 to completely inhibit vacuole formation in response to VacA may reflect the fact that approximately 20–30% of cells fail to switch on expression of dominant negative Rac1 when doxycycline is removed.

Although it is clear that Rac1 regulates the activity of VacA in epithelial cells, the mechanism by which this occurs is not yet clear. The localization of wild type GFP-Rac1 around the vacuoles induced by VacA might point to a role in the formation of these structures. The VacA-induced vacuoles are known to be hybrid late endosomal-lysosomal compartments, and it is possible that Rac might be involved in lysosomal biogenesis (3). However, a clear role for another GTPase, Rab7, has been established in the late endosomal-lysosomal fusion events that occur following attachment of H. pylori (32, 33). Rab7 acts intracellularly, the mechanism of entry is far from clear. The localization of wild type GFP-Rac1 around the vacuoles induced by VacA might point to a role in the formation of these structures. The VacA-induced vacuoles are known to be hybrid late endosomal-lysosomal compartments, and it is possible that Rab7 might be involved in lysosomal biogenesis (3). However, a clear role for another GTPase, Rac1, has been established (6). Alternatively, it might be that Rac1 is regulating the uptake of VacA across the plasma membrane, although, while there is good evidence that VacA acts intracellularly, the mechanism of entry is far from clear (29).

Even though this is the first report describing a role for Rac1 in regulating VacA activity, it is interesting to note that 12-O-tetradecanoylphorbol-13-acetate, which has been reported to increase the sensitivity of cells to VacA (30), is a potent stimulator of Rac-dependent signaling events (27, 31). The observation that members of the Rho family play important roles in the pathogenesis of a number of other micro-organisms, including L. monocytogenes and S. flexneri, has generated a great deal of interest in understanding how micro-organisms regulate intracellular signaling events (16, 17). Clearly it will be important to establish the signaling events that occur following attachment of H. pylori, and it is important to note there are significant distinctions between the mechanisms of infection employed by S. flexneri and L. monocytogenes and those employed by H. pylori. Both Shigella and Listeria effect entry into, or movement within and between, epithelial cells by regulating the actin cytoskeleton via members of the Rho family. In contrast, Helicobacter infection does not involve entry into epithelial cells, but rather it attaches to the apical surface of epithelial cells where it is protected from the acidic environment of the gut by the mucus lining. Interestingly, there is evidence for reorganization of the actin cytoskeleton at sites of attachment, and there is some evidence that this is an event regulated by members of the Rho family (32, 33).

In summary, we have demonstrated that activity of VacA, the major virulence factor of H. pylori, is regulated by the Rac1 GTPase, raising the possibility that other noninvasive pathogens might employ similar mechanisms.

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FIG. 3. VacA-induced vacuole formation is regulated by Rac1. MDCK epithelial cells expressing myc epitope-tagged constitutively active (A–D) and dominant negative (E–H) Rac1 under the control of the tetracycline repressible trans-activator were cultured for 16 h in the absence of doxycycline to induce expression of the Rac1 mutants. In C–H, 200 ng ml$^{-1}$ VacA was added, and cells were cultured for a further 4 h. In A and B, cells were cultured exactly as for C–H but in the absence of VacA. Cells were fixed in phosphate-buffered saline containing 4% paraformaldehyde, and vacuole formation was assessed by phase microscopy (A, C, E, and G). Expression of the Rac1 mutants was verified by indirect immunofluorescent staining of cells using an antibody to the myc epitope (B, D, F, and H).

FIG. 4. Quantitative analysis of VacA-induced vacuole formation in MDCK cells expressing constitutively active and dominant negative Rac1. Vacuole formation in MDCK cells was quantitated by monitoring the release of Neutral Red dye following treatment of cells expressing constitutively active (V12) or dominant negative (N17) Rac with VacA. Cells were cultured in 96-well plates in triplicate wells for 16 h in the presence or absence of doxycycline before the addition of 200 ng ml$^{-1}$ VacA for a further 4 h. Uptake of Neutral Red dye into acidic vacuoles was quantitated on a microtiter plate reader, and results were corrected for differences in cell numbers. Data presented here are the mean and standard error from three separate experiments.

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