Alteration of Epithelial Structure and Function Associated with PtdIns(4,5)P_2 Degradation by a Bacterial Phosphatase

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Elucidation of the role of PtdIns(4,5)P_2 in epithelial function has been hampered by the inability to selectively manipulate the cellular content of this phosphoinositide. Here we report that SigD, a phosphatase derived from Salmonella, can effectively hydrolyze PtdIns(4,5)P_2, generating PtdIns(5)P. When expressed by microinjecting cDNA into epithelial cells forming confluent monolayers, wild-type SigD induced striking morphological and functional changes that were not mimicked by a phosphatase-deficient SigD mutant (C462S). Depletion of PtdIns(4,5)P_2 in intact SigD-injected cells was verified by detachment from the membrane of the pleckstrin homology domain of phospholipase C β, used as a probe for the phosphoinositide by conjugation to green fluorescent protein. Single-cell measurements of cytosolic pH indicated that the Na⁺/H⁺ exchange activity of epithelia was markedly inhibited by depletion of PtdIns(4,5)P_2. Similarly, anion permeability, measured using two different halide-sensitive probes, was depressed in cells expressing SigD. Depletion of PtdIns(4,5)P_2 was associated with marked alterations in the actin cytoskeleton and its association with the plasma membrane. The junctional complexes surrounding the injected cells gradually opened and the PtdIns(4,5)P_2-depleted cells eventually detached from the monolayer, which underwent rapid restitution. Similar observations were made in intestinal and renal epithelial cultures. In addition to its effects on phosphoinositides, SigD has been shown to convert inositol 1,3,4,5,6-pentakisphosphate (IP_5) into inositol 1,4,5,6-tetrakisphosphate (IP_4), and the latter has been postulated to mediate the diarrhea caused by Salmonella. However, the effects of SigD on epithelial cells were not mimicked by microinjection of IP_4. In contrast, the cytoskeletal and ion transport effects were replicated by hydrolyzing PtdIns(4,5)P_2 with a membrane-targeted 5-phosphatase or by occluding the inositide using high-avidity tandem PH domain constructs. We therefore suggest that opening of the tight junctions and inhibition of Na⁺/H⁺ exchange caused by PtdIns(4,5)P_2 hydrolysis combine to account, at least in part, for the fluid loss observed during Salmonella-induced diarrhea.

INTRODUCTION

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) has long been recognized as an important source of second messengers. Hydrolysis of PtdIns(4,5)P_2 by phospholipase C yields diacylglycerol, a potent activator of most protein kinase C isoforms and other enzymes bearing C1 domains, and inositol 1,4,5-trisphosphate, which induces release of calcium stored in the endoplasmic reticulum (Taylor, 2002). In addition, phosphorylation of PtdIns(4,5)P_2 by class I phosphatidylinositol 3-kinases generates phosphatidylinositol 3,4,5-trisphosphate, a ligand and activator of various effectors that contain pleckstrin homology (PH) domains (Vanhasebroeck et al., 2001; Lemmon, 2003). Not only are its metabolites critical for signal transduction, but PtdIns(4,5)P_2 itself serves multiple regulatory functions in the cell. It affects several stages of actin microfilament assembly and remodeling, including uncapping of barbed ends, severing and bundling of filaments, and de novo nucleation (Hilpela et al., 2004; Roth, 2004). In addition, several studies have shown that a variety of ion channels and exchangers are directly modulated by the local concentration of PtdIns(4,5)P_2 (Leung et al., 2000; Hilgemann et al., 2001; Hilgemann, 2003).

The functional importance of the metabolites generated from PtdIns(4,5)P_2 has been convincingly established by pharmacological means. Potent and reasonably

Abbreviations used in this paper: DAPI, 4',6-diamidino-2-phenyl-indole; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; HPMI, HEPES-buffered solution RPMI-1640; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,4,5,6-tetrakisphosphate; IP_5, inositol 1,3,4,5,6-pentakisphosphate; MQAE, N-(chloroacrylonamyl)-6-methoxyquinolinium bromide; NHE, Na⁺/H⁺ exchanger; PH, pleckstrin homology; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; TLC, thin layer chromatography.

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specific phospholipase C and protein kinase C inhibitors are available, which have been used to evaluate the physiological role of diacylglycerol, inositol 1,4,5-trisphosphate (IP₃), and their effectors (Botelho et al., 2000; Matsui et al., 2001; Spitaler and Cantrell, 2004). Similarly, class I phosphatidylinositol 3-kinases can be selectively inhibited by wortmannin or LY294002 to assess the effects of phosphatidylinositol 3,4,5-trisphosphate biosynthesis (Vieira et al., 2001; Djordjevic and Driscoll, 2002). By contrast, establishing the function of PtdIns(4,5)P₂ has proven to be considerably more difficult. No specific inhibitors of the kinases that generate this phosphoinositide have been described, and the coexistence of multiple kinase isoforms and splice variants has precluded genetic analysis. Definitive confirmation of the involvement of PtdIns(4,5)P₂ in the regulation of ion channels and transporters stems largely from electrophysiological studies in excised patches or perfused cells, where the cytosolic aspect of the membrane can be accessed directly by solutions containing varying amounts of PtdIns(4,5)P₂ or bacterial lipases (Estacion et al., 2001; Loussouarn et al., 2003; Oliver et al., 2004). The multiple actions of PtdIns(4,5)P₂ in cytoskeletal dynamics have been similarly gleaned primarily from studies of disrupted cells (Nebl et al., 2000; Hsin-Yi et al., 2004).

Remarkably little is known about the role of PtdIns(4,5)P₂ in epithelial structure and function. The barrier and vectorial transport functions of epithelia are eminently dependent on the maintenance of the integrity of individual cells and of their intercellular contacts. This requirement rules out the use of most of the techniques that have been successfully applied to study PtdIns(4,5)P₂ function in other systems. Because molecular or genetic manipulation of the kinases that generate PtdIns(4,5)P₂ is subject to the limitations described above, we considered instead the possibility of modulating the cellular PtdIns(4,5)P₂ content by expression of phosphoinositide-specific phosphatases. Some success has been reported using Inp54p, a yeast inositol polyphosphate 5′-phosphatase (Raucher et al., 2000). In our hands, however, this enzyme, as well as the native forms of the mammalian phosphoinositide phosphatases synaptojanin, SKIP and OCRL, had negligible effects on the PtdIns(4,5)P₂ content of epithelial cells (unpublished data). Failure of the phosphatases to target to the plasmalemma and/or to become activated likely account for these observations.

We reported that SigD/SopB, an injected virulence factor of Salmonella species, altered the binding of a PtdIns(4,5)P₂-specific PH domain to the inner leaflet of the plasma membrane in HeLa cells (Terebiznik et al., 2002). We now present evidence that SigD/SopB (referred to hereafter as SigD) functions as a 4′-phosphatase that dephosphorylates PtdIns(4,5)P₂ to form PI(5)P. By cloning this bacterial phosphatase into a mammalian expression vector we were able to introduce it by micro-injection into intact epithelia, which are notoriously refractory to transfection. This strategy enabled us to analyze the consequences of selective depletion of PtdIns(4,5)P₂ in confluent epithelia. Because Salmonella is an enteric pathogen that injects SigD along with several other products into host epithelial cells via a type III secretion system encoded by the Salmonella pathogenicity island (SPI)-1 (Galan, 1998), we focused our study primarily on IEC-18 cells, a line derived from the rat small intestine (Ma et al., 1992). In this manner, we simultaneously learned about the possible consequences of Salmonella infection on intestinal physiology.

MATERIALS AND METHODS

Materials and Solutions

IP₃ was purchased fromMatreya Inc. Biochemicals. Rhodamine-phalloidin, 4′,6-diamidino-2-phenylindole (DAPI), FM4-64, SNARF-5F, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), and Alexa 568 microinjection dye were from Molecular Probes. Radiolabeled [32P]yATP and [3H]-myoinositol were from MP Biomedicals (formerly ICN Biomedicals). The Na⁺/H⁺ exchanger (NHE) inhibitors HOE694 and S3226 were gifts of Hoechst and Aventis, respectively. FBS, α-modified Earle’s medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), HEPES-buffered solution RPMI-1640 (HPMI), PBS, and penicillin plus streptomycin were from Wisent. Antibodies to ZO-1 were purchased from Zymed Laboratories. Monoclonal antibodies against active caspase 3 were from BD Biosciences. Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. All other reagents were from Sigma-Aldrich. Microinjection buffer consisted of (in mM) 140 KCl plus 10 HEPES, pH 7.4 at 37°C and was filter sterilized before use. Na⁺-rich solution contained 130 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 20 HEPES, pH 7.4 at 37°C. In K⁺-rich solution, all NaCl was replaced by KCl, and in Cl⁻-depleted solution NaCl was replaced by NaO₃.

Cell Culture

HeLa, IEC-18, and OK cells were obtained from the American Tissue Culture Collection. IEC-18 cells were grown in DMEM supplemented with 5% FBS and 0.1 U/ml bovine pancreatic insulin. HeLa cells were grown in DMEM supplemented with 10% FBS. OK cells were grown in α-MEM supplemented with 10% FBS. All cells were maintained under 5% CO₂ at 37°C. When required, cells were pretreated with 100 μM LY294002 for 30 min and the inhibitor was kept in the medium throughout the experiment.

DNA Constructs

The mammalian expression vectors encoding wild-type SigD and the inactive SigD mutant (C462S) have been described elsewhere (Marcus et al., 2001). The vector pEGFP:PLCδ-PH encodes the PH domain of PLCδ fused to EGFP (PLCδ-PH-EGFP), pGFP::PM encodes the myristoylation/palmitoylation sequence from Lyn fused to GFP (PM-GFP). Both of these were the gift of T. Meyer (Stanford University, Stanford, CA) and their construction has been described before (Teruel et al., 1999). 2FYVE-GFP consists of two tandem FYVE domains from EEA1 conjugated to GFP and has been described previously (Vieira et al. 2001). The 2(PLCδ-PH)-GFP construct was made by combining two tandem PLCδ-PH domains to GFP and was a gift of M. Rebecca (State University of New York, Stonybrook, NY). The phosphatase domain of mammalian synaptojanin 2 fused to a CAAX box modeled after the carboxy terminal sequence of K-Ras (PD-CAAX) was the gift of...
the samples using HPLC.

### Microinjection Protocol

Cells were grown on 25-mm glass coverslips and used for experiments 2 d after the monolayer had reached confluence. The coverslips were then transferred to a thermostatted Leiden chamber and incubated with HPMI medium supplemented with antibiotic/antimycotic mixture (1:500) for 30 min before microinjection. The microinjection solution contained 50 μg/ml of the indicated mixture of plasmids, typically a 5 to 1 ratio of SigD to PLC6-PH4-GFP cDNA. Microinjection was performed under phase contrast microscopy using an Eppendorf Transjector 5246 controlled by an Eppendorf 5171 Micromanipulator. Microinjected cells were identified by the expression of the fluorescent protein products. To minimize evaporation during prolonged observation periods the chambers were sealed using a second coverslip secured with a small amount of silicon grease.

### Cytosolic pH Determinations

For cytosolic pH determinations IEC-18 cells were microinjected with cDNA encoding YFP (with or without SigD cDNA). Cells were next incubated for 3 h at 37°C under 5% CO₂ to allow expression of the proteins. Alternatively, the cells were loaded with SNARF-5F by loading with 20 μM of the precursor acetoxyethyl ester for 30 min. The coverslips were then mounted in a thermostatted Leiden holder, bathed in a Na⁺-rich buffer, and placed on the stage of a Leica fluorescence microscope equipped with a PL Fluotar 100×/1.30 N.A. oil immersion objective. Sutter filter wheels positioned excitation and emission filters in front of a Hg lamp and the acquisition camera, respectively. For YFP, excitation was at 480 nm and was directed to the cells through a 510-nm dichroic mirror. Emitted fluorescence was selected through a 535/525-nm filter. For SNARF-5F, excitation was at 550 nm and emission was recorded at 580 and 640 nm. Images were captured with an Orca CR cooled charge-coupled device camera (Hamamatsu). Image acquisition was controlled by the Metafluor software v3.5 (Universal Imaging Corp.). To determine background, an area identical to the region of interest was selected outside the transfected cell and fluorescence was acquired. At the end of the experiment, a calibration curve of fluorescence (ratio) vs. pH was obtained in situ by sequential perfusion with K⁺-containing medium buffered to predetermined pH values (between 6.0 and 7.4) containing 10 μg/ml nigericin. Calibration curves were constructed by plotting the extracellular pH, assumed to be identical to the cytosolic pH under these conditions, against the corresponding fluorescence.

To determine the relative contribution of specific NHE isoforms, pH determinations were performed in the presence of 1 μM HOE694 to selectively inhibit NHE1, 20 μM HOE694, to inhibit both NHE1 and NHE2, or 20 μM HOE694 plus 5 μM of S226, to inhibit the three main plasmalemmal isoforms.

### Determinations of Anion Permeability

Anion permeability was estimated by two methods: first, the anion-sensitive variant of YFP (H148Q) was used by a microfluorimetric method similar to that described above for pH determinations. Because YFP derivatives are also inherently sensitive to pH, a second series of experiments was performed using a chloride-sensitive dye, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), which was loaded into the epithelial cells by hypotonic stress, following expression of SigD or the PD-CAAX domain of the transfected construct. To identify the cells expressing the phosphatase, SigD was cotransfected with PLC6-PH4-GFP. By studying the presence and distribution of this construct we not only identified the transfectants, but ensured that the phosphatase had in fact exerted its effects on PtdIns(4,5)P₂. Control experiments were performed in cells expressing PLC6-PH4-GFP only. After loading hypotonically with MQAE, cells were allowed to recover in isotonic medium for 30 min before measurement of anion permeability.

### Table I

Analysis of Phosphoinositide Content after Infection

<table>
<thead>
<tr>
<th>Phosphoinositide</th>
<th>Uninfected</th>
<th>Salmonella Wild Type</th>
<th>Salmonella ΔsigD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>8.2 ± 0.7%</td>
<td>5.5 ± 0.4%</td>
<td>6.9 ± 0.4%</td>
</tr>
<tr>
<td>PtdInsP</td>
<td>5.5 ± 0.7%</td>
<td>7.4 ± 0.9%</td>
<td>3.5 ± 0.2%</td>
</tr>
</tbody>
</table>

Phosphoinositide levels were quantified before (Uninfected) and after infection of HeLa cells with either wild-type or ΔsigD deficient (ΔsigD) Salmonella. Lipids were extracted and analyzed by HPLC as detailed in Materials and Methods. The amount of the phosphoinositides is given as percent of PtdIns in the same sample. PtdInsP refers to the sum of both PtdIns(4)P and PtdIns(5)P, which are not resolved by the chromatographic system used. Data are means ± SEM of three separate experiments.
Calibration was made using 10 μM nigericin and 10 μM tributyltin chloride in K⁺-rich media of varying Cl⁻ concentration.

Staining with Fluorescent Markers and Confocal Microscopy
Cells grown on coverslips were washed twice with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for at least 30 min, followed by quenching of excess fixative with 100 mM glycine for 10 min. Cells were blocked while permeabilized using 5% skimmed milk in PBS containing 0.1% Triton X-100 for 60 min. ZO-1 was immunostained using a polyclonal primary antibody (1:100), followed by secondary Cy3-conjugated donkey anti-rabbit antibodies (1:1,000).

To label F-actin, fixed and permeabilized cells were stained with a 1:500 dilution of rhodamine-phalloidin for 30 min. Two different methods were used for assessment of apoptosis. In both cases the cells were fixed with 3% paraformaldehyde for 15 min at room temperature and, where indicated, permeabilized with 0.1% saponin in PBS. For caspase-3 staining the preparation was blocked for 30 min in medium containing 10% donkey serum in 0.1% saponin in PBS. For caspase-3 staining the preparation was incubated with antibody to active caspase-3 (1:200), followed by fluorescently conjugated secondary antibodies, each for 60 min. ZO-1 was immunostained using a polyclonal primary antibody (1:100), followed by secondary Cy3-conjugated donkey anti-rabbit antibodies (1:1,000).

Results were consistent with the notion that SigD actively dephosphorylates PtdIns(4,5)P₂ to PtdInsP. This was confirmed by analyzing the PtdInsP content of the samples (Table I). In untreated cells the major PtdInsP peak detected by HPLC, which is comprised of PtdIns(4)P plus PtdIns(5)P, constituted 5.5% of the PtdIns. Infection with bacteria expressing SigD resulted in a considerable increase in PtdInsP, to 7.4% of PtdIns. Notice that the magnitude of the increase in PtdInsP is similar to the decrease in PtdIns(4,5)P₂ recorded under the same conditions, suggesting a precursor and product relationship. Importantly, the PtdInsP content was not increased when the cells were infected with SigD-deficient bacteria (Table I), confirming that the phosphatase is responsible for the generation of the inositide.

SigD Generates Phosphatidylinositol 5-Phosphate
Because SigD has homology to mammalian inositol 4-phosphatasas (Norris et al. 1998), the disappearance of PtdIns(4,5)P₂ is likely the result of its conversion to phosphatidylinositol 5-phosphate (PtdIns(5)P). While the preceding data confirmed the near stoichiometric disappearance of PtdIns(4,5)P₂ and concomitant
appearance of PtdInsP, the HPLC system used is unable to differentiate PtdIns(5)P from phosphatidylinositol 4-phosphate (PtdIns(4)P). To test whether PtdIns(5)P is fact formed we used a combined enzymatic and TLC assay that involves conversion of PtdIns(5)P to PtdIns(4,5)P_2 by PtdIns(4,5)P_2 4-kinase. The identity of the product of the enzymatic reaction was validated by HPLC (for details see Materials and methods and Morris et al. [2000] and Niebuhr et al. [2002]). As above, to discern the contribution of SigD we compared the effects of wild-type Salmonella to those of mutants devoid of SigD. The deletion of sigD was verified by immunoblotting (Fig. 1 B). As shown in Fig. 1 A, infection with wild-type, but not SigD-deficient Salmonella, generated a sizable amount of PtdIns(5)P, indicated by the formation of PtdIns(4,5)P_2. That this difference between the two bacterial strains was due to the deletion of sigD was confirmed by reintroduction of the phosphatase into the deficient Salmonella. Transformation of (ΔsigD) Salmonella with a plasmid encoding wild-type SigD restored the production of PtdIns(5)P upon infection (Fig. 1 A). Like other active phosphatases, SigD has an essential cysteine in its active site. We generated a plasmid encoding a mutant SigD where the critical cysteine was replaced by serine, namely SigD (C462S). SigD-deficient bacteria were then transformed with this plasmid and used to infect mammalian cells. Immunoblotting was used to confirm that the level of expression of the mutant and wild-type forms of SigD was similar in the strains used (Fig. 1 B). Unlike the plasmid encoding wild-type SigD, the C462S mutant plasmid was unable to restore the appearance of PtdIns(5)P (Fig. 1 C), confirming that the 4-phosphatase activity of the enzyme is responsible, at least in part, for the hydrolysis of PtdIns(4,5)P_2.

In principle, PtdIns(5)P could have also been generated by dephosphorylation of PtdIns(3,5)P_2 on position 3, as has been reported for myotubularins (Tronchere et al., 2004), or by sequential dual dephosphorylation of PtdIns(3,4,5)P_3, a preferred substrate of SigD in vitro (Norris et al., 1998). Several lines of evidence argue against these possibilities. First, analysis by HPLC revealed that the cellular content of PtdIns(3,5)P_2 is far too low to account for the increase in PtdInsP recorded in the same experiments. It is unlikely that PtdIns(3,5)P_2 is rapidly generated by other processes and simultaneously degraded by SigD, thus failing to accumulate. This is indicated by the finding that the PtdIns(3,5)P_2 content of cells infected with SigD-deficient bacteria is also extremely low, similar to that of untreated or wild-type Salmonella-infected cells (Fig. S1 A). Like PtdIns(3,5)P_2, the content of PtdIns(3,4,5)P_3 in resting cells is much too low (≈0.1% of PtdIns) to account for the formation of PtdInsP, did not decrease upon infection with wild-type bacteria, and did not increase in cells infected with (ΔsigD) bacteria (unpublished data).

Additional evidence against the involvement of PtdIns(3,5)P_2 or PtdIns(3,4,5)P_3 in the generation of PtdIns(5)P was obtained using LY294002. This compound effectively inhibits both class I and class III PtdIns 3 kinases, which are required for the formation

Figure 1. Generation of PtdIns(5)P by SigD. HeLa cells were infected with the indicated strains of Salmonella and, after 10 min at 37°C, the reaction was stopped and lipids were extracted as described in Materials and methods. The PtdIns(5)P content was next analyzed by its enzymatic conversion to radiolabeled PtdIns(4,5)P_2 by insertion of the gamma phosphate of [32P]ATP, catalyzed by the type II phosphoinositide kinase, which selectively phosphorylates the 4'-position of PtdIns(5)P. The resulting lipids were next separated by thin layer chromatography. A representative chromatogram is shown in A. The site where the lipid mixture was spotted (Origin) and the position of the PtdIns(4,5)P_2 produced are indicated. The cells were infected, from left to right, with wild-type Salmonella, SigD-deficient Salmonella, SigD-deficient Salmonella bearing a plasmid expressing wild-type SigD cDNA, and SigD-deficient Salmonella bearing a plasmid expressing the catalytically inactive SigD mutant SigD (C462S). Lipids from uninfected cells are shown in the last lane. The deletion of SigD and the level of expression of the retransformed wild-type and mutant SigD were verified by immunoblotting. Identical amounts of bacteria were loaded, and lanes from the same gel and exposure are shown in B, which is representative of two similar blots. Lanes were separated in the image to facilitate alignment with the TLC (above) and bar graph (below). Molecular mass markers (in kD) are shown to the right. In C the amount of PtdIns(4,5)P_2 present in each TLC sample was quantified by HPLC and online continuous-flow liquid-scintillation counting. The chromatogram and quantitation shown are representative of three similar independent experiments.
of PtdIns(3,4,5)P$_3$ and of PtdIns(3)P, the precursor of PtdIns(3,5)P$_2$, respectively. Fig. S1 B shows that under the conditions used, LY294002 blocked PtdIns 3-kinase activity; endosomal PtdIns(3)P, detected using a tandem FYVE domain from EEA1, disappeared upon treatment with the drug (panels B and B′). When cells were first treated with LY294002 and then infected with wild-type *Salmonella* in the presence of the inhibitor, the generation of PtdIns(5)P persisted (Fig. S1 C).

Jointly, these observations provide convincing evidence that SigD from *Salmonella* is an effective phosphoinositide phosphatase capable of converting PtdIns(4,5)P$_2$ to PtdIns(5)P. Whether the increase in PtdInsP measured by HPLC can be accounted for in its entirety by PtdIns(5)P remains to be defined, since the combined enzymatic/TLC assay does not yield quantitative estimates. Nevertheless, it is clear that at least a fraction of the PtdInsP generated is PtdIns(5)P.

### Effect of SigD on the PtdIns(4,5)P$_2$ Content of Epithelial Cells

The IEC-18 cell line was used as an epithelial model for these studies for two reasons. First, it forms well-defined polarized monolayers with distinct junctional complexes (see below). Second, IEC-18 cells were derived from the small intestine, where *Salmonella* infection occurs in humans. Thus, our studies could potentially yield both basic and pathophysiological information. Confluent epithelial monolayers are notoriously difficult to transfect, and this was found to be the case for IEC-18 cells as well (unpublished data). To introduce cDNA into these cells without disrupting the integrity of the monolayer we opted instead to use microinjection. The number of cells that can be injected precluded biochemical analyses but provided reproducible results when single cells were analyzed microscopically.

The PH domain of PLCδ has been used extensively to monitor PtdIns(4,5)P$_2$ in live cells (Stauffer et al., 1998; Raucher et al., 2000; Varnai et al., 2002). The distribution of PLCδ-PH-GFP expressed in IEC-18 cells is illustrated in Fig. 2 (A–C). As anticipated from its association with PtdIns(4,5)P$_2$, the construct is mostly bound to the inner aspect of the plasma membrane. Both the apical and basolateral membranes were comparably labeled (see z-axis reconstruction in Fig. 2 A′). The plasmalemmal association of PLCδ-PH-GFP is best visualized by confocal microscopy (Fig. 2 A) but can be readily appreciated also by conventional fluorescence microscopy (Fig. 2 C). Note that microinjection and expression of PLCδ-PH-GFP had no discernible effect on...
IEC-18 cell morphology, as is evident from DIC microscopy (Fig. 2 B).

The effect of SigD on PLCδ-PH-GFP distribution is shown in Fig. 2 (D–F). As early as 1 h after microinjection the phosphatase reverted the association of PLCδ-PH-GFP with the membrane, rendering the construct largely cytosolic. The displacement was evident both by confocal (Fig. 2 D) and conventional microscopy (Fig. 2 F). Line scans of images like those in Fig. 2 (A and D), followed by densitometry of the resulting scans were used to quantify the reproducibility and statistical significance of the effect of SigD. The pixel density at the membrane, defined by staining with FM4-64, was normalized to that of the cytosol. In seven similar determinations the membrane-to-cytosol ratio averaged 7.97 ± 1.4, while in SigD-treated cells it was 0.97 ± 0.01 (data are mean values ± SEM). This difference is highly significant (P < 0.001). DIC imaging of SigD-injected cells revealed that they underwent extensive vacuolation, and fluorescence microscopy indicated that these vacuoles are sealed, as they excluded PLCδ-PH-GFP.

The effects of SigD were not restricted to intestinal epithelial cells, but were observed also in renal epithelial cells. The accumulation of PLCδ-PH-GFP at the plasmalemma and its displacement by SigD were noted also in OK cells, an opossum kidney line (see Fig. S2). Together, the results of Fig. 2 and Fig. S2 confirm the effectiveness of SigD as a phosphoinositide phosphatase when introduced into mammalian cells and highlight its ability to deplete PtdIns(4,5)P2 in epithelial cells.

Effect of SigD on Epithelial Morphology and Integrity

In addition to the vacuolation reported in Fig. 2, other structural changes were consistently noted in cells expressing SigD. These changes developed gradually over time and were always preceded by displacement of PLCδ-PH-GFP from the membrane, suggesting that they were a consequence of the hydrolysis of PtdIns(4,5)P2. The development of the structural changes is illustrated in Fig. 3. Concomitantly with the appearance of vacuoles, cells injected with SigD extend lamellipodia beyond the original junctional complexes (see Fig. 3, B and B', and Fig. 4 below). At later times blebbing of the apical surface is apparent (Fig. 3, C and C'). These blebs extend well beyond the surface of the monolayer and are in fact best detected when the focal plane of the microscope is raised by 10 μm above the normal apical surface (Figs. 3, H and H'). Eventually, most of the PtdIns(4,5)P2-depleted cell bulges above the monolayer, rounding up (Fig. 3, D–I) and finally detaching from the epithelium. All of these effects depend on the
phosphatase activity of SigD, since they were never observed in cells expressing SigD(C462S). As shown in Fig. 3 (E and J), such cells retained normal morphology and remained as integral components of the monolayer even after 5 h, when cells expressing wild-type SigD had generally bulged and rounded up.

Because PtdIns(4,5)P₂ effectively modulates the actin cytoskeleton, we speculated that the morphological changes induced by SigD were due, at least in part, to alterations in actin filament structure. To test this notion cells were fixed and permeabilized at various times after microinjection with cDNA encoding SigD and PLCδ-PH-GFP. The cells were then stained with rhodamine-phalloidin to reveal F-actin and analyzed by confocal microscopy (Fig. 4). These experiments indicated that actin underwent a biphasic change. At the early stages of action of SigD, the F-actin content of the cells seemingly increased, particularly in the lamellipodia that extended beyond the normal junctional boundaries (Fig. 4, A and B). At this stage, the SigD-injected cells remained within the context of the monolayer, though the stress fibers attaching them to the substratum were somewhat depleted (not depicted). Subsequently, bulging above the monolayer and vacuolation became apparent (panels C and D, labeled “mid-stage” in Fig. 4). At this time the lamellipodia had receded and the net F-actin content of the cells had diminished. At even later stages the vacuoles were resorbed and the cells rounded up and protruded above the monolayer (Fig. 4, E and F, labeled “late stage”), ultimately detaching. Little F-actin remained at this stage, accounting for cell rounding. Of note, the neighboring cells rapidly occupied the space vacated by the PtdIns(4,5)P₂-depleted cell, a form of epithelial restitution.

The Effects of SigD on the Cytoskeleton Are Mediated by Depletion of PtdIns(4,5)P₂
As described above, the structural changes elicited by SigD were not mimicked by SigD(C462S). Because this inactive mutant also failed to alter PtdIns(4,5)P₂, depletion of the inositide is likely responsible for the observed structural changes. However, it is also possible that accumulation of PtdIns(5)P is involved and, since SigD hydrolyzes several soluble inositol phosphates (Norris et al., 1998), generation of IP₄ from IP₃ is another potential cause of the morphological changes.

Figure 4. Effect of PtdIns(4,5)P₂ depletion on F-actin and PtdIns(4,5)P₂ distribution. Intestinal epithelial cells (IEC-18) were microinjected with PLCδ-PH-GFP and SigD cDNA (1:5 ratio). The monolayers were incubated for varying periods of time and were then fixed, permeabilized, and stained with rhodamine-phalloidin to reveal F-actin. The stained cells were then analyzed by confocal fluorescence microscopy. Panels A–F show PLCδ-PH-GFP in green and F-actin in red. Panels A′–F′ show only F-actin in white. Panels A, A′, C, C′, E, and E′ illustrate representative x vs. y confocal slices acquired near the middle of the cell, while B, B′, D, D′, F, and F′ are the corresponding x vs. z reconstructions. A and B are representative of cells fixed at an early stage, i.e., 2–4 h after injection. C and D are representative of cells fixed at an intermediate stage, i.e., 4–5 h after injection. E and F are representative of cells fixed at a late stage, i.e., 5–6 h after injection. The images shown are representative of 10 similar experiments of each type. Bar, 10 μm.
The latter possibility was analyzed by directly microinjecting the cells with IP₄. The concentration of IP₄ in the injection pipette was 100 μM. Because we estimate that the injection volume approximates 5–10% of the total cell volume, a final concentration of 5–10 μM IP₄ must have been delivered to the cells. This concentration is higher than the concentration of IP₄ reported in cells infected by *Salmonella* (Zhou et al., 2001). In four experiments, injection of IP₄ had no discernible effect on IEC-18 cell morphology or association with the monolayer (unpublished data). Similarly, microinjection of PtdIns(5)P or extracellular addition of this lipid in the presence of carriers that facilitate intracellular delivery of inositides was without effect on cell morphology (unpublished data).

These observations suggest that depletion of PtdIns(4,5)P₂, not production of PtdIns(5)P or IP₄, was the cause of the morphological changes. To validate this conclusion, we manipulated PtdIns(4,5)P₂ by two independent procedures. First, we transfected a phosphoinositide phosphatase of mammalian origin, synaptojanin-2. To improve the efficiency of hydrolysis, a construct encoding the phosphatase domain was targeted to the membrane by addition of a prenylation motif, a polycationic sequence and CAAX box modeled after the C terminus of K-Ras. Expression of this construct effectively displaced PLCδ-PH-GFP from the membrane (Fig. 5 A; see Fig. 2 A for comparison with untreated control), indicating hydrolysis of PtdIns(4,5)P₂. More prolonged expression of the synaptojanin-2 phosphatase-CAAX construct induced cell blebbing (Fig. 5 B) and eventual detachment of the cells from the substratum. In parallel, the actin cytoskeleton was drastically altered, with marked disappearance of stress fibers (Fig. 5 C).

We also tested the effects of overexpression of an avid PtdIns(4,5)P₂ ligand, which at sufficiently high concentrations should mask a significant fraction of the inositol. To this end we used a construct of two tandem copies of the PH domain of PLCδ, which is predicted to have enhanced avidity for PtdIns(4,5)P₂. As shown in Fig. 5 D, 2(PLCδ-PH)-GFP binds very effectively to the plasmalemmal PtdIns(4,5)P₂ and, when expressed at high levels, induced cell blebbing (Fig. 5, D and E) and pronounced changes in cytoskeletal architecture (Fig. 5 F). Because the structural changes produced by synaptojanin-2 phosphatase-CAAX and 2(PLCδ-PH)-GFP resemble those induced by SigD, we believe that diminution in the amount of available plasmalemmal PtdIns(4,5)P₂ is the common underlying mechanism.

**Effect of PtdIns(4,5)P₂ Depletion on Tight Junction Integrity**

Since tight junctions are necessary to maintain epithelial integrity, which was lost upon expression of SigD, we suspected that depletion of PtdIns(4,5)P₂ may have destabilized the junctional complexes. This was tested by staining control and transfected monolayers with antibodies to ZO-1, a well-established tight junction marker. As reported earlier (Ma et al., 1992), in confluent monolayers of IEC-18 cells ZO-1 was found to line the cellular junctions in a virtually continuous pattern (Fig. 6 A). Similar results were obtained in cells injected only with soluble or membrane-associated GFP constructs used as indicators of expression (not illustrated). Even at the early stages of the PtdIns(4,5)P₂ depletion process
(1–3 h after injection) disruption of the junctional integrity was apparent, as judged by the discontinuities in the ZO-1-staining pattern (Fig. 6, B and C). Preferential staining with ZO-1 at the cell boundary was completely eliminated at later stages. A disruption of ZO-1 architecture was also observed in cells expressing the synaptotagmin-CAAX construct as well as in cells with high levels of 2(PLCδ-PH)-GFP expression. Importantly, the effect of SigD injection on junctional integrity was absent when the phosphatase-inactive mutant SigD(C462S) was used (unpublished data), pointing to depletion of PtdIns(4,5)P$_2$ as the underlying mechanism.

Does SigD Induce Apoptosis of Epithelial Cells?
The blebbing and rounding observed in cells expressing SigD is also characteristic of apoptotic cells (Majno and Joris, 1995). Moreover, infection with Salmonella has been reported to promote apoptosis of some cell types (Knodler and Finlay, 2001). It was therefore important to establish whether depletion of PtdIns(4,5)P$_2$ by SigD sufficed to trigger programmed cell death in IEC-18 cells. To this end, cells were coinjected with SigD or SigD(C462S) and a fluorescent protein, used as an injection and expression marker. Apoptosis was initially assessed from nuclear morphology in cells stained with DAPI. A very small fraction of the uninjected cells (1–2%) had an apoptotic phenotype, consistent with findings in other cells (Majno and Joris, 1995). The sensitivity of the detection procedure was validated treating the cells with 100 nM of staurosporine for 2 or 5 h, which increased the apoptotic index to 13.4% and 34.9%, respectively. More importantly, when comparing over 100 cells from three experiments no significant increase in the fraction of apoptotic cells was detected in SigD-expressing cells (2%), even at the longest times tested. Longer times were not investigated, as the cells tended to detach from the monolayer (see Fig. 3).

DAPI staining is simple, yet not the most sensitive method for detection of apoptosis. Gross changes in nuclear morphology occur only in advanced stages of apoptosis and the time window of our experiments may have been insufficient to reach such stages. For this reason, we also assessed apoptosis using a more sensitive method that detects earlier stages of programmed cell death. Control and SigD-expressing cells were stained with a specific antibody that recognizes the activated form of caspase-3, an essential early component of the apoptotic chain. As in the case of DAPI, no significant difference in the fraction of apoptotic profiles was measured in four determinations between control and SigD-expressing cells (2% vs. 1%) while distinct apoptosis was observed following a short treatment with staurosporine (10.5% after 2 h). Jointly, these experiments indicate that apoptosis is not prevalent during the first 4–6 h of expression of SigD, at a time when PtdIns(4,5)P$_2$ is extensively degraded and cell morphology is drastically altered. These results are consistent with the findings of Santos et al. (2001), who determined that SigD was not required for the induction of apoptosis in host cells in an animal model of Salmonella infection.

Effect of PtdIns(4,5)P$_2$ Depletion on Anion Permeability
In the context of the intestinal epithelium, transient opening of the junctional complexes would be predicted to result in loss of vectorial ion transport and possibly cause diarrhea (Uzzau and Fasano, 2000). Indeed,
SigD has been identified as a principal factor in causing diarrhea in animal models of *Salmonella* infection (Galyov et al., 1997). However, the loss of fluid was not attributed to loss of junctional integrity, but was instead proposed to be caused by increased Cl\(^{-}\) secretion in response to elevated levels of IP\(_4\) (Norris et al., 1998). This conclusion, however, was derived from studies of chloride influx into nonepithelial (HEK293) cells and required validation in epithelia.

We implemented measurements of anion permeability using a novel halide-responsive variant of the yellow fluorescent protein, YFP(H148Q). This protein responds to variations in halide concentration with changes in pK\(_{a}\) that, at constant pH, translate into fluorescence changes (Jayaraman et al., 2000). IEC-18 cells were microinjected with the cDNA encoding YFP(H148Q), together with or without SigD. A typical measurement is illustrated in Fig. 7 A, where the concentration of Cl\(^{-}\) in the medium was varied stepwise, by isoosmotic replacement with I\(^{-}\). The latter anion is a more effective quencher of YFP(H148Q) emission, resulting in a progressive diminution of fluorescence. The loss was largely, but not entirely reversible, due to photobleaching incurred during repeated acquisitions. Indeed, a comparable partial decrease was noted upon repeated illumination at constant [Cl\(^{-}\)]. The bleaching component could be readily interpolated and corrected in our measurements. Finally, tributyltin and nigericin were used to calibrate the fluorescence vs. [Cl\(^{-}\)]. Using this approach, we were able to compare the rates of halide exchange in control cells and in cells expressing SigD. Cells were tested at the early and mid stages of expression, but not at the late stages (see Fig. 3), because the tenuous attachment of the latter to the monolayer made measurements unreliable and calibration impossible. The data collected from 11 determinations are summarized in Fig. 7 B. Contrary to the predictions made on the basis of the work of Feng et al. (2001), we found that halide permeability was in fact depressed by SigD.

Because GFP-derived probes such as YFP(H148Q) can be affected by environmental parameters other than the halide concentration, including the pH, it was imperative to ascertain that this unexpected discrepancy did not result from an experimental artifact. We therefore performed an independent set of experiments using a
different chloride-sensitive probe, MQAE. While sensitive to halide concentrations, MQAE fluorescence is not altered by the physiological anions $\text{HCO}_3^-$, $\text{SO}_4^{2-}$, and $\text{PO}_4^{3-}$, by cations, or by pH (Verkman et al., 1989). Results of a typical experiment are shown in Fig. 7 C and the summary of 10 determinations from three separate experiments is presented in Fig. 7 D. As found using YFP (H148Q), the MQAE results indicate that anion permeability is diminished by treatment with SigD. To ensure that this diminution was caused by hydrolysis of PtdIns(4,5)P$_2$, as opposed to dephosphorylation of inositol polyphosphates, we also tested the effects of PD-CAAX on chloride permeability. As shown in Fig. 7 D (gray bar), this membrane-associated, phosphoinositide-specific phosphatase produced an inhibition comparable to that seen with SigD, ruling out mediation by hydrolysis of soluble inositol polyphosphates. These findings are not consistent with elevated Cl$^-$ secretion as the mechanism underlying SigD-induced diarrhea and, in addition, suggest a role for PtdIns(4,5)P$_2$ in the modulation of epithelial anion transport.

**Effect of PtdIns(4,5)P$_2$ Depletion on Na$^+$/H$^+$ Exchange**

The apical membranes of intestinal and renal epithelial cells are active sites of Na$^+$ resorption. Much of this absorption occurs in exchange for H$^+$ and is coupled to Cl$^-$/$\text{HCO}_3^-$ exchange and to osmotically obliged fluid absorption. There is evidence in model systems that some isoforms of the NHE are sensitive to the concentration of PtdIns(4,5)P$_2$ (Aharonovitz et al., 2000; Fuster et al., 2004), though this has not been validated for epithelial cells. The possible regulation of epithelial exchangers by PtdIns(4,5)P$_2$ is important not only in the context of Salmonella infection, but also because PtdIns(4,5)P$_2$ can vary during signaling, changes in cell volume, and ischemia. We therefore took advantage of the selective phosphatase activity of SigD and PD-CAAX to investigate the specific effects of PtdIns(4,5)P$_2$ depletion on Na$^+$/H$^+$ exchange activity in epithelial cells.

Epithelial cells often coexpress multiple isoforms of NHE. To our knowledge, the isoforms present in IEC-18 cells have not been defined. Therefore, we first assessed the overall NHE activity of these cells and estimated the contribution of the "housekeeping" NHE1 isoform and of specialized isoforms like NHE2 and NHE3. NHE activity was measured as the rate of sodium-induced pH recovery, measured over the first 1–2 min. Data show rates of alkalinization and are means ± SEM of at least three experiments of each type.
The contribution of individual isoforms to this response was investigated using HOE694, an inhibitor that differentially affects various NHE isoforms. At low (≤1 μM) concentrations, HOE694 selectively blocks NHE1, while at higher concentrations (20 μM) it also blocks NHE2. Under these conditions, NHE3 is largely unaffected (Counillon et al., 1995). A different inhibitor has the converse effect; at 5 μM S3226 preferentially inhibits NHE3. As shown in Fig. 8 A, 82% of Na+/H+ exchange in IEC-18 cells was blocked by 1 μM HOE694. The fractional inhibition was not increased when 20 μM HOE694 was used, but an additional 11% was inhibited when 5 μM S3226 was added. These findings imply that Na+/H+ exchange in IEC-18 cells is largely mediated by NHE1, with a smaller contribution by NHE3. No NHE2 activity was detectable in these cells.

We proceeded to assess the effect of PtdIns(4,5)P2 depletion on exchange activity. As for Cl− determinations, cells were tested at early and mid stages of expression, but not at the late stages, because the tenuous attachment of the latter to the monolayer made calibration impossible. Indeed, late stage cells become permeant to H+ equivalents and could not be acid loaded to the same degree as control cells. As illustrated in Fig. 8 (B and C), depletion of PtdIns(4,5)P2 by expression of SigD produced inhibition of NHE activity, which became more pronounced at the later stages of expression of this phosphatase. A significant inhibition of the antiport was also observed when cells were transfected with PD-CAAX, confirming that physiological PtdIns(4,5)P2 levels are required for optimal NHE activity. These findings are in accord with the inositol sensitivity reported in nonpathelial model systems expressing NHE1 (Aharonovitz et al., 2000; Fuster et al., 2004) and NHE3 (Fuster et al., 2004).

DISCUSSION

SigD Is a PtdIns(4,5)P2 Phosphatase

The biological activity of SigD was first inferred from its sequence homology with mammalian phosphoinositol phosphatases (Norris et al., 1998). Its ability to dephosphorylate inositol phosphates was then demonstrated in vitro and cells expressing SigD were found to accumulate IP3. It was subsequently appreciated, however, that SigD is largely membrane bound (Marcus et al., 2002), not an ideal location for the degradation of soluble substrates. Its subcellular location suggested that the degradation of inositol phosphates may not be the sole or even the most important function of SigD and prompted investigators to test whether it can additionally hydrolyze phosphoinositides. This notion was tested by in vitro experiments that were met with positive, yet conflicting, results; different groups confirmed that SigD is active against phospholipids, but the substrate selectivity varied among reports (Norris et al. 1998; Marcus et al., 2001; Hernandez et al., 2004).

The first indication that SigD effectively cleaves phospholipids when introduced into mammalian cells was provided by Terebiznik et al. (2002), who described dissociation of PLC6-PH-GFP from the cytosolic face of the membrane in cells infected by wild-type, but not SigD-deficient, Salmonella. More recently, the appearance of PtdIns(3)P in Salmonella-containing vacuoles was also attributed to the lipid phosphatase activity of SigD (Hernandez et al., 2004). Here we provide direct biochemical evidence of phosphoinositide conversion induced by SigD. Cells infected by Salmonella were found to have a markedly elevated content of 4' and/or 5'-phosphorylated PtdInsP, which corresponded quantitatively to the concomitant decrease in PtdIns(4,5)P2.

At least a fraction of the product was PtdIns(5)P, a lipid that is expressed in minute amounts in untreated cells. The increase in PtdIns(5)P induced by SigD is strongly reminiscent of the effect of IpgD, a Shigella virulence factor that bears considerable sequence homology (38.5%) with SigD. IpgD concomitantly produced depletion of PtdIns(4,5)P2, pointing to dephosphorylation of this inositide at the 4’ position as the source of PtdIns(5)P. Indeed, like SigD, IpgD bears a region of homology with mammalian inositol 4-phosphatases (Norris et al., 1998). Thus, both the Shigella and Salmonella phosphatases appear to target primarily PtdIns(4,5)P2 and, as such, are very useful tools to study the functional requirement for this phosphoinositide in animal cells.

Tools for the Manipulation of PtdIns(4,5)P2 in Intact Mammalian Cells

While phospholipids were initially thought to serve mainly a structural role, they are now believed to participate in signaling, vesicular traffic, and cytoskeleton regulation. Some of these suggested functions have been deduced from in vitro experiments but require verification in intact cells. Such verification has been most difficult, largely because selective manipulation of specific inositides has proven extremely complex. In the case of PtdIns(4,5)P2, several strategies have been attempted. Metabolic inhibition has been found to be paralleled by depletion of PtdIns(4,5)P2. However, the specificity of this manipulation is questionable, in view of the myriad ATP-dependent events in cells. PtdIns(4,5)P2 is often depleted acutely by activation of phospholipase C using calcium ionophores (Varani and Balla, 1998). As in the case of ATP depletion, however, the pleiotropic effects of calcium elevation make assignment of functional consequences ambiguous. More specific effects can, in principle, be obtained using phosphoinositide-specific phosphatases. Several 5-phosphatases have been identified and reported to cleave PtdIns(4,5)P2. We have tested heterologous overexpression of OCRL and SKIP, with negligible effects on PtdIns(4,5)P2 content.
(unpublished data). This may be attributed to the subcellular location of these enzymes, which are found mostly in endomembranes (Gurung et al., 2003; Ungewickell et al., 2004). In our hands, heterologous expression of Inp54p, a 5-phosphatase from yeast, had only minor effects on epithelial cells. We were similarly disappointed by the effects of overexpressed full-length synaptojanin (unpublished data), even though this phosphatase is found at the plasma membrane (McPherson et al., 1996).

It is conceivable that synaptojanin, and possibly the other 5-phosphatases as well, require the coexpression of ancillary factors and/or are regulated by heretofore unidentified mechanisms.

A constitutively active phosphatase that does not require cofactors and is not subject to regulation would be more suitable to acutely manipulate PtdIns(4,5)P2. Our observations suggest that bacterial 4-phosphatases, such as SigD and IpgD, fulfill these requirements and have ample activity to induce massive degradation of PtdIns(4,5)P2 in a variety of mammalian cells. These enzymes can be expressed heterologously in eukaryotic cells by introduction of the available cDNAs, and recombinant proteins could in principle be used for microinjection when even more acute effects are desired. Unlike the synaptojanin-CAAX construct, the bacterial phosphatases do not require posttranslational modifications, which facilitates recombinant expression and accelerates generation of the active protein in transfected cells. It must be borne in mind that both SigD and IpgD generate PtdIns(5)P in the plasmalemma as they degrade PtdIns(4,5)P2 and that this product may have biological activity, although none has been described to date and we failed to observe any effects following intracellular delivery of PtdIns(5)P. Nevertheless, a bioactive role for PtdIns(5)P cannot be ruled out.

Effects of PtdIns(4,5)P2 Depletion on Epithelial Structure and Function

Expression of SigD was accompanied by marked changes in the structure of epithelial cells. These were likely caused by the depletion of plasmalemmal PtdIns(4,5)P2, since neither PtdIns(5)P nor IP4, the known products of SigD activity, were able to recapitulate the structural changes. Evidence that PtdIns(4,5)P2 depletion can alter the actin cytoskeleton was provided by Raucher et al. (2000), who used optical tweezers to measure membrane tether forces in nonpolarized cells expressing Inp54p, a 5-phosphatase from yeast. Our results expressing synaptojanin-CAAX and 2(PLCδ-PH)-GFP are also consistent with a requirement for normal levels of PtdIns(4,5)P2 to maintain intact the epithelial structure.

The effects of PtdIns(4,5)P2 depletion on cytoskeletal architecture followed a peculiar time course. At the earliest stages, the cells emitted apical lamellipodial extensions, and this coincided with extensive vacuolation. Subsequently, stress fibers disappeared gradually as the overall F-actin content diminished and the cells ultimately underwent blebbing and rounding as they protruded from the monolayer. Inactivation of ezrin likely contributed to disruption of the apical structure, since this protein is thought to anchor transmembrane proteins to actin fibers in a manner that is stimulated by PtdIns(4,5)P2 (Bretscher et al., 2002). The perinuclear actin band was also depleted upon hydrolysis of PtdIns(4,5)P2 and this was associated with loss ofZO-1 staining, consistent with junctional uncoupling. The loss of cell–cell contacts can readily explain the extrusion of the SigD-transfected cells from the context of the epithelial monolayer. To our knowledge, this is the first evidence that tight junctional integrity depends on the availability of PtdIns(4,5)P2.

We also assessed the consequences of PtdIns(4,5)P2 depletion on a limited number of physiological parameters characteristic of epithelia. First, we noted that Na+/H+ exchange was severely depressed by reducing the level of the phosphoinositide. These observations are in good agreement with earlier reports in non-epithelial model systems (Aharonovitz et al., 2000; Fuster et al., 2004) and emphasize the multiplicity of actions of PtdIns(4,5)P2. It remains unclear whether the effects of the phospholipid are exerted directly or via intermediate proteins like ezrin (Baumgartner et al., 2004). In any event, it is clear that conditions predicted to alter PtdIns(4,5)P2, such as ischemia or infection by Salmonella or Shigella, will be accompanied by reduced salt and water absorption and may thus contribute to overall fluid loss during diarrhea (see below).

Contribution of PtdIns(4,5)P2 Loss to Diarrhea Induced by Pathogenic Bacteria

The occurrence of diarrhea is one of the major clinical complications of infection by Salmonella. Targeted deletion experiments initially demonstrated that the bacterial factors responsible for the fluid loss resided in the first pathogenicity island of the Salmonella genome (SPI 1). Further refinements of the deletional analysis indicated that the effector proteins SipA, SopA, SigD/SopB, SopD, and SopE2 act in concert to induce diarrhea (Zhang et al., 2002). The individual contribution of each of these proteins has not been thoroughly defined, but SigD is believed to be a major effector and has therefore been studied in most detail. In particular, it has been proposed that SigD contributes to fluid loss by stimulation of intestinal chloride secretion, and this effect was postulated to be mediated by IP4 (Feng et al., 2001). The rationale for this proposal is based on the earlier report that PtdIns(3,4,5)P3 acts as an inhibitor of calcium-dependent chloride secretion (Eckmann et al., 1997). By competing with PtdIns(3,4,5)P3, IP4 was proposed to relieve this inhibition, unmasking chloride secretion. In support of this hypothesis, Feng et al. (2001) reported that expression of SigD in HEK293
cells promoted an increase in chloride permeability. However, this hypothesis appears unlikely on the following grounds. (a) Chloride secretion is not constitutive and requires an increase in cytosolic calcium above the basal level. Indeed, the inhibitory effect of PtdIns(3,4,5)P$_3$ is exerted on the secretion induced by muscarinic agents, but is not expected to occur in otherwise unstimulated cells. (b) The purported inhibitory effect of IP$_4$ requires competition with existing PtdIns(3,4,5)P$_3$. However, the resting levels of apical PtdIns(3,4,5)P$_3$ in unstimulated cells are very low, insufficient to raise basal calcium by activation of phospholipase Cy (Melendez et al., 1999) or of Tec-family kinases, if present (Carpenter, 2004). (c) Shigella, which is related to Salmonella and produces a similar diarrhea, was reported by Eckmann et al. (1997) not to generate IP$_4$. (d) Lastly, the reported effects of SigD on chloride permeability appear not to be universal and, importantly, were not observed in intestinal epithelial cells in our experiments. Jointly, these considerations detract from the weight that should be given to IP$_4$ and chloride secretion as contributing factors to Salmonella-induced diarrhea.

The experiments presented here suggest alternative mechanisms. First, depletion of PtdIns(3,4,5)P$_3$ by SigD resulted in inhibition of Na$^+$/H$^+$ exchange activity, which is predicted to depress the rate of sodium and water absorption (by inhibition of NHE3) and compromise intracellular pH homeostasis (by inhibition of NHE1). At later stages, cells expressing SigD lost F-actin and underwent opening of their junctional complexes. The ensuing transient increase in transepithelial conductance could readily account for decreased fluid absorption. Indeed, junctional opening has been invoked in the etiology of diarrhea in both bacterial and viral infections (Dickman et al., 2000; Goosney et al., 2000; Bertelsen et al., 2004). Clearly, though informative, microinjection of SigD into IEC-18 cells is not a perfect model of bacterial infection. This paradigm is useful in that it enabled us to isolate the contribution of SigD to the bacterial effector phenotype. On the other hand, neither the intensity nor the duration of the effects of expressed SigD are likely to be identical to those it exerts during bacterial infection. In the heterologous expression system SigD is likely to localize to both apical and basolateral membranes, which may not be the case during infection. Lastly, though derived from the rat ileum (Ma et al., 1992), IEC-18 cells are not perfect mimics of the primary epithelium. Nevertheless, the information obtained revealed the potential of SigD to exert a variety of biological effects, the contribution of which to the pathogenicity of the bacteria will need to be validated in vivo.

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