Regulation of Salmonella-induced Neutrophil Transmigration by Epithelial ADP-ribosylation Factor 6*

Alison K. Criss‡§, Milton Silva†, James E. Casanova**, and Beth A. McCormick†

From the ‡Department of Cell Biology, University of Virginia, Charlottesville, Virginia 22908, the §Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, Massachusetts 02115, and the **Combined Program in Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital, Charlestown, Massachusetts 02129

Salmonella typhimurium elicits an acute inflammatory response in the host intestinal epithelium, characterized by the movement of polymorphonuclear leukocytes (PMN) across the epithelial monolayer to the intestinal lumen. It was recently shown that SipA, a protein secreted by S. typhimurium, is necessary and sufficient to drive PMN transmigration across model intestinal epithelia (Lee, C. A., Silva, M., Sber, A. M., Kelly, A. J., Galyov, E., and McCormick, B. A. (2000) Proc. Natl. Acad. Sci. USA 97, 12283–12288). However, the epithelial factors responsible for this process have not been identified. Here, for the first time, we demonstrate that S. typhimurium-induced PMN transmigration across Madin-Darby canine kidney-polarized monolayers is regulated by the GTPase ARF6. Apically added S. typhimurium promoted the translocation of ARF6 and its exchange factor ARNO to the apical surface. Overexpression of a dominant-negative mutant of ARF6 inhibited Salmonella-induced PMN transmigration, which was due to a reduction in apical release of the PMN chemoattractant PEEC (pathogen-elicited epithelial chemoattractant), without affecting bacterial internalization. Furthermore, ARF6 and its effector phospholipase D (PLD) were both required for bacteria-induced translocation of protein kinase C (PKC) to membranes. These results describe a novel signal transduction pathway, in which Salmonella initiates an ARF6- and PLD-dependent lipid signaling cascade that, in turn, directs activation of PKC, release of PEEC, and subsequent transepithelial PMN movement.

Bacteria of the Salmonella species cause diseases in humans and other animals ranging from gastroenteritis (S. enteritidis, S. typhimurium) to enteric fever (S. typhi, S. paratyphi). Salmonella contacting the luminal surface of the intestinal epithelium activate a bacterial type III protein secretion apparatus, encoded on a 40-kb segment of the bacterial chromosome (1). This pathogenicity island-1-secreted virulence factors (e.g., SopE/E2, SptP, SopB/SigD, and others) are subsequently translocated into the enterocyte, where they modulate host protein activity and lipid production, resulting in apical membrane ruffling and concomitant bacterial internalization (1).

Salmonella typhimurium evokes a potent inflammatory response in the host, the hallmark of which is the migration of polymorphonuclear leukocytes (PMN) across the intestinal epithelium to the luminal surface (3). This process can be separated into three distinct phases: (a) extravasation of circulating PMN from the microvasculature; (b) passage of PMN across the lamina propria to a subepithelial space; (c) paracellular movement of PMN across the epithelial monolayer. PMN recruitment is coordinated by the epithelial release of an array of proinflammatory cytokines, among which are two potent PMN chemoattractants. Interleukin-8 (IL-8), which is secreted primarily at the basolateral surface, establishes a gradient across the lamina propria that guides PMN to the basal aspect of enterocytes (4, 5). Subsequent PMN transit through the epithelial monolayer to the luminal surface (“PMN transmigration”) is directed by an apically released chemokine termed epithelial chemoattractant (PEEC) (6). Modelling of this final, rate-limiting step of PMN movement in vitro demonstrated that PMN transmigration could occur independently of bacterial internalization, although both were dependent on the SPI-1 secretion apparatus (7). Consistent with this observation, the S. typhimurium SPI-1-secreted product SipA was recently found to be both necessary and sufficient for induction of PMN transmigration across model intestinal epithelia but not for bacterial entry, in a protein kinase C (PKC)-dependent manner (8).

PMN transmigration only occurs following apical, but not basolateral application of S. typhimurium (7), implying that host factors functioning at the apical domain of epithelial cells modulate this event. One regulatory protein that satisfies this requirement is ARF6, a member of the ARF subgroup of GTPases of the Ras superfamily. ARF proteins were first defined as cofactors necessary for the cholera toxin-catalyzed ADP-ribosylation of the αs subunit of heterotrimeric G proteins (9). Class I (ARF 1–3) and class II (ARF 4–5) ARF proteins localize to the Golgi apparatus as well as endosomes, where they regulate vesicle budding by inducing the assembly of coat proteins such as coatamer protein I and clathrin onto the membrane; salt solution; IL-8, interleukin-8; MDCK, Madin-Darby canine kidney; PA, phosphatidic acid; PAP, phosphohydrolase; PC, phosphatidylcholine; PEEC, pathogen-elicited epithelial chemoattractant; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; MAPK, mitogen-activated protein kinase; ARF6, ADP-ribosylation factor 6.

* This work was supported in part by National Institutes of Health Grants DK-56754 and DK-35056 (to B. A. M.) and DK-58536 and AI-32991 (to J. E. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† A National Science Foundation predoctoral fellow.

‡ To whom correspondence should be addressed: Dept. of Cell Biology, Box 800732, University of Virginia Health Sciences Center, Charlottesville, Virginia 22906-0732. Tel.: 434-243-4821; Fax: 434-982-3912; E-mail: jec9e@virginia.edu.

§ The abbreviations used are: SPI-1, Salmonella pathogenicity island-1; DAG, diacylglycerol; FMLP, N-formylmethionylleucylphenylalanine; GEF, guanine nucleotide exchange factor; HBSS, Hank’s balanced salt solution; IL-8, interleukin-8; MDCK, Madin-Darby canine kidney; PA, phosphatidic acid; PAP, phosphohydrolase; PC, phosphatidylcholine; PEEC, pathogen-elicited epithelial chemoattractant; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; MAPK, mitogen-activated protein kinase; ARF6, ADP-ribosylation factor 6.

This paper is available on line at http://www.jbc.org

Received for publication, July 23, 2001, and in revised form, October 4, 2001
Vol. 276, No. 51, Issue of December 21, pp. 48431–48439, 2001
Published, JBC Papers in Press, October 18, 2001, DOI 10.1074/jbc.M106969200

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
brane surface (10). ARF6, the only class III ARF, is novel in its localization to the plasma membrane and endosomal structures in non-polarized cells (11, 12). Interestingly, ARF6 is highly expressed in polarized epithelial cells, where it localizes primarily to the apical brush border and apical early endosomes (13, 14). ARF6 has been implicated in endocytosis and membrane recycling of a subset of membrane proteins, as well as in remodeling of the cortical actin cytoskeleton (11, 15–18). In addition, ARF6 appears to be a key signal transducer downstream of G protein-coupled receptors and receptor tyrosine kinases (19–22). As with other GTPases, ARFs cycle between active (GTP-bound) and inactive (GDP-bound) states, which are facilitated by accessory proteins. Activation of ARFs is stimulated by guanine nucleotide-exchange factors (GEFs), which promote release of GDP and binding of GTP, whereas inactivation is stimulated by GTPase-activating proteins. One GEF for ARF proteins, ARNO, has also been detected at the apical domain of polarized epithelial cells, where it colocalizes with ARF6 (23–25).

Previous work from our laboratory has investigated differences in the responses of polarized epithelial cells to S. typhimurium depending on whether the bacteria were applied to the apical or basolateral surface (7, 26). Here, we investigate the role of the small GTPase ARF6 in S. typhimurium-epithelial cell interactions using the polarizing MDCK epithelial cell line as a model system. Our results suggest that ARF6 regulates a phospholipase D-dependent lipid signaling cascade that is necessary for recruitment of protein kinase C to the apical cell surface and, subsequently, for Salmonella-induced PMN transmigration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The T23 clone of MDCK strain II cells (27), which stably express the tetracycline-repressible transactivator (28), was cultured as previously described (26). Cell culture inserts were established by seeding T23 cells at confluent density on Transwell filter supports (Costar) and used 4–5 days later. Collagen-coated cell culture inverts of T23 monolayers for PMN transmigration experiments were generated as previously described (5, 29). T84 cells were cultured as described previously (5).

The ARF6/N48R construct (a gift of T. Leto, National Institutes of Health, Bethesda, MD) was subcloned into the pAdTet adenoviral expression vector and recombinant adenovirus produced as previously described (13). ARNO and another ARF6 adenoviruses have been previously described (30). 18 h prior to the experiment, T23 monolayers were placed in Hank’s balanced salt solution lacking calcium and magnesium (HBSS; Life Technologies, Inc.) and their apical surface exposed to ARNO or ARF6 adenovirus at a titr sufficient to infect 95% of cells in the population. 2 h later, cells were replaced in growth medium in the presence of 0.5 ng/ml doxycycline (Sigma Chemical Co.) to permit attenuated gene expression. Under these conditions, expression of ARF6 constructs was found to be 1- to 2-fold over endogenous ARF6. Infected cells maintained in 20 ng/ml doxycycline to completely repress expression were used as controls. Monolayer integrity was assessed prior to each experiment by measuring transepithelial resistance with an EVOM epithelial voltohmeter (World Precision Instruments); resistances were used as controls. Membrane integrity was assessed prior to each experiment by measuring transepithelial resistance with an EVOM epithelial voltohmeter (World Precision Instruments); resistances were used as controls.

**Bacterial Strains**—The wild-type S. typhimurium strain SL1344 and its isogenic derivatives VV341 and EE633, which are disrupted in the hilA and sipA loci, respectively, have been previously described (8, 31). To produce invasion-competent *Salmonella*, bacteria were grown under O2-limited conditions as originally described by Lee et al. (32). In some experiments, to promote synchronous, rapid internalization of bacteria, *Salmonella* were cultured as described by Méresse et al. (33). Under both conditions, bacterial cultures corresponded regularly to 5 × 10⁸ colony forming units/ml. Colony forming units were determined by plating appropriate dilutions of the cell lysates on MacConkey agar (Difco). Invasion assays were performed as described previously, with O2-limited bacteria (26).

**PMN Transmigration Assay**—Human PMN were isolated from normal volunteers and purified as previously described (7). The basolateral-to-apical PMN transmigration assay was performed with T23 inverted monolayers essentially as described for T84 cells (7), and transmigrated PMN were quantified as described previously (29). In each experiment, *Salmonella*-induced PMN transmigration was compared with the positive control, transmigration in response to the chemotactant N-formylmethionylleucylphenylalanine (fMLP; 10 nM), and to the negative controls, HBSS alone or hilA+ Salmonella (5). Salmonella-induced transmigration assays were followed as previously described (26). Endogenous ARF6 was detected using a polyclonal antibody (gift of V. Hsu, Harvard Medical School, Boston, MA) followed by Cy2-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch). Myc-ARNO was detected using the monoclonal 9E10 antibody followed by Cy2-labeled donkey anti-mouse IgG (Jackson ImmunoResearch). Bacteria were detected using either a rabbit polyclonal anti-Salmonella antibody (H antigen, poly A-Z, 1:500, Difco) followed by Texas-Red labeled donkey anti-rabbit IgG (Jackson ImmunoResearch) or a monoclonal IgA antibody to *Salmonella* (Sal4, gift of M. Neutra, Harvard Medical School) and rhodamine-labeled anti-mouse IgA (E-Y Laboratories). Classical PKC was detected using a monoclonal anti-PKC (αβγ, Upstate Biotechnology Inc.) followed by Texas Red-labeled anti-ακβγ IgG; bacteria were simultaneously recognized by the monoclonal Sal4 antibody followed by fluorescein-labeled anti-mouse IgA (Southern Biotechnology Associates). Images were obtained with a Zeiss LSM 410 confocal laser scanning microscope.

**PKC Translocation Assay**—Movement of PKC to a detergent-insoluble membrane fraction was assayed as described in Ferro et al. (35). All supernatants were concentrated over a 10-kDa molecular mass cutoff filter (Centricron-10, Amicon). In subsequent experiments, the intermediate (buffer B) step of the Ferro et al. protocol was omitted, thereby yielding cytosolic and membrane subcellular fractions. 50 µg of each fraction was separated on 10% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted for classical PKC isoforms (Uspate Biotechnology Inc.) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (AP Biotech). Treatments with inhibitors or with adenosine analogs did not affect the relative distribution of protein between cytosolic and membrane fractions. PLD1- and PLD2-specific polyclonal antisera were gifts of S. Bourguin. SipA-HA protein was purified as previously described (8) and added to the apical surface at a concentration of 20 µg/ml. Because of day-to-day differences, basal values of membrane-associated PKC were found to vary. The results presented are representative immunoblots from at least three independent experiments, and within each experiment, the percentage of translocated PKC is presented relative to the uninfected control.
membrane ruffling and particle phagocytosis in some cell types (15, 17, 18, 36), we examined whether ARF6 participated in the internalization of *S. typhimurium* by epithelial cells. Polarized monolayers of the tetracycline-responsive T23 MDCK cell line were induced to express wild-type or dominant-negative (T27N) ARF6 by means of a recombinant adenovirus expression system (see “Experimental Procedures”). In this cell line, the presence of 20 ng/ml doxycycline in the culture medium represses gene expression, whereas its removal stimulates it. Coupling of an adenoviral delivery system with tetracycline-regulated transgene expression facilitates the overexpression of proteins in polarized epithelial cells, which are otherwise refractory to conventional transfection techniques. ARF6-expressing monolayers were exposed to *Salmonella*, and the resulting invasion efficiency was calculated. As seen in Fig. 1B, expression of dominant-negative ARF6(T27N) did not inhibit bacterial internalization at the apical pole of MDCK cells. Furthermore, ARF6(T27N) also did not affect bacterial entry from the basolateral cell surface or in the non-polarized HeLa cell line. We therefore conclude that ARF6 is dispensable for internalization of *Salmonella* in epithelial cells.

**Regulation of *S. typhimurium*-induced Transepithelial PMN Movement by ARF6**—Although ARF6 did not affect bacterial entry, we investigated whether it might regulate another signaling process critical to *S. typhimurium* pathogenesis, the basolateral-to-apical migration of PMN across the intestinal epithelium. We initially established that canine epithelial cells (MDCK cells) were capable of promoting the transmigration of human PMN in response to *S. typhimurium*. Polarized monolayers of MDCK cells or T84 cells (a human polarizing epithelial cell line commonly used in PMN transmigration studies (5)) were first presented with invasive bacteria at the apical cell surface. PMN were then added to the basolateral aspect of the cells, and migration of PMN across the epithelium to the apical chamber was measured as described under “Experimental Procedures.” MDCK cells promoted the physiological (basolateral-to-apical) movement of PMN in response to *S. typhimurium* to nearly the same extent as T84 cells (Fig. 2A). Furthermore, a strain of *S. typhimurium* lacking a functional SPI-1 type III secretion apparatus (*hilAΔ*), which renders it invasion-deficient, was unable to promote significant PMN transmigration across MDCK monolayers (Fig. 2A). These observations indicate that MDCK cells, like human intestinal epithelial cells, can selectively induce proinflammatory signaling cascades in response to bacterial invasion.

We then used the adenovirus-mediated gene expression system described above to assess the role of ARF6 in *S. typhimurium* signaling to basolaterally situated PMN. Expression of ARF6(T27N) significantly attenuated *Salmonella*-induced PMN transmigration compared with control (virally infected, doxycycline-repressed) cells (Fig. 2B). PMN transmigration was modestly increased by wild-type ARF6 in a reproducible, but not statistically significant, manner (1.3-fold increase in transmigrated PMN for wild-type ARF6 versus control, p = 0.07). Conversely, activation of ARF6 via expression of the exchange factor ARNO potently stimulated *Salmonella*-induced PMN transmigration (Fig. 2C), without affecting apical bacterial entry. 2 The exchange activity of ARNO was required for this effect: Expression of a catalytically inactive point mutant of ARNO (E156K) produced a consistent reduction in PMN transmigration across control monolayers, p = 0.095. Notably, expression of a mutant of ARF6 (Q67L) predicted to be locked in the GTP-bound state did not promote this increase in PMN transmigration, suggesting that cycling of the GTPase is critical to transduction of the *Salmonella*-induced signal. 2 Expression of these constructs did

---

2 A. Criss and J. Casanova, unpublished observations.
Epithelial ARF6 Regulates Salmonella-induced PMN Migration

**Fig. 2. Requirement for ARF6 in S. typhimurium-induced PMN transmigration across epithelial monolayers.** A, transmigration of human PMN across canine MDCK monolayers in response to S. typhimurium. Monolayers of T23 cells (hatched bars) or the human polarizing epithelial cell line T84 (black bars) were infected apically with SL1344 (WT) or the invasion-deficient hiLA strain (inv-), then exposed to human PMN. PMN transversing the monolayers in a basolateral-to-apical direction were quantified as described under “Experimental Procedures.” B, PMN transmigration in response to the positive control, PMN-chemoattractant FMLP; (−), PMN transmigration across cells maintained in HBSS. Results are expressed as PMN cell equivalents (CE) relative to a PMN standard curve and are the means ± S.D. of three independent filters. B, inhibition of PMN transmigration by ARF6(T27N). Monolayers of T23 cells inducibly expressing wild-type (WT) or dominant-negative (T27N) ARF6 were infected with SL1344, and PMN transmigration was measured as in A. Control, ARF6(T27N) adenovirus-infected cells in the presence of 20 ng/ml doxycycline to repress expression. *, p = 0.012 (Student’s t test versus control). C, stimulation of PMN transmigration by the ARF6 GEF ARNO. The PMN transmigration assay was performed with T23 cells expressing wild-type (ARNO) or catalytically inactive (E156K) ARNO. Control, ARNO adenovirus-infected cells in the presence of 20 ng/ml doxycycline. **, p = 0.001 (versus control).

not affect background levels of PMN transmigration, i.e. in cells presented with either HBSS or hiLA Salmonella, nor did it affect PMN transmigration in response to FMLP, a potent PMN chemoattractant, whose action in this setting does not require or induce epithelial cell signaling.2 Taken together, these results strongly suggest that ARF6 regulates Salmonella-induced PMN transmigration in epithelial monolayers, independent of an effect on bacterial internalization.

**Modulation of PMN Transmigration by ARF6 Is Due to an Effect on Release of the Chemokine PEEC—**To shed light on the mechanism by which ARF6 modulates S. typhimurium-induced PMN transmigration, we analyzed the effect of expression of ARF6(T27N) on two PMN chemotactic agents released by infected epithelial cells, PEEC and IL-8. PEEC release was measured using a functional assay quantifying PMN transmigration in response to a semi-purified, concentrated apical supernatant from Salmonella-infected cells.6 As shown in Fig. 3A, PEEC-containing supernatant from infected cells expressing ARF6(T27N) exhibited 40% less PMN transmigration than supernatant from cells not expressing the construct. This reduction was similar in magnitude to the effect of ARF6(T27N) on PMN transmigration when assayed directly (Fig. 2B). Our result is consistent with previous work demonstrating that PEEC is the primary chemokine responsible for PMN transmigration in this in vitro system.5, 6.

We then examined the effect of ARF6(T27N) on release of IL-8 to the basolateral medium. Expression of ARF6(T27N) did not prevent Salmonella-induced IL-8 secretion, as detected by immunoblot, and instead modestly potentiated its release relative to the virally infected but non-expressing control (compare lanes 3 and 4, Fig. 3B). ARF6(T27N) also had no effect on IL-8 mRNA levels.2 Importantly, none of the ARF6 and ARNO constructs, including ARF6(T27N), had an effect on polarized protein secretion, or on proper targeting of membrane proteins to the appropriate epithelial surface.2 We therefore conclude that the block in Salmonella-induced PMN transmigration by ARF6(T27N) is specifically due to a reduction in apical release of the chemoattractant PEEC.

S. typhimurium Invasion Induces the Translocation of PKC to a Membrane Fraction—Previously, S. typhimurium-induced PMN transmigration, through the bacterial effector SipA, was shown to require the catalytic activity of PKC (8). Because ARF6 and PKC can operate together in signaling pathways (15, 37), we investigated whether the regulation of PMN transmigration by ARF6 involved PKC activity. First, we examined the subcellular distribution of PKC during apical Salmonella invasion. Salmonella induced the redistribution of classical isoforms of PKC to a Triton X-100-insoluble membrane preparation, with translocation of PKC at 60 min of invasion exceeding...
that in response to the phorbol ester PMA, a direct PKC activator (Fig. 4A). Similarly, apical addition of recombinant SipA protein promoted movement of PKC into a total membrane fraction (both Triton X-100-soluble and insoluble membranes) with a potency similar to wild-type Salmonella (Fig. 4B), indicating that SipA alone recapitulates signals connecting the bacteria to PKC. Conversely, a strain of Salmonella deleted in the sipA locus was unable to induce membrane translocation of PKC.\(^2\) Comparable results from these experiments were obtained in the T84 model system.\(^3\) Translocation of PKC from cytosol to membranes is associated with activation of the enzyme, and accordingly, preliminary results from substrate phosphorylation assays indicate that a subset of classical and novel PKC isoforms became active during Salmonella-apical membrane interaction.\(^4\) Furthermore, immunofluorescence microscopy of Salmonella-infected MDCK monolayers demonstrated that the membrane translocation of PKC observed by immunoblot reflected its redistribution to the apical plasma membrane, specifically to sites of active bacterial invasion (Fig. 4C). Translocation of PKC to the apical membrane in response to SipA protein has also been observed.\(^4\) Having shown that ARF6 was recruited to sites of bacteria-host cell interaction at the apical plasma membrane (Fig. 1A), we examined whether ARF6 was necessary for the Salmonella-induced partitioning of PKC to membranes. As shown in Fig. 5, expression of ARF6(T27N) completely prevented Salmonella from promoting membrane translocation of PKC. We conclude that the activation of PKC required for bacteria-induced PMN transmigration is regulated by ARF6.

**Phospholipase D Is Stimulated during Salmonella Invasion and Is Required for PMN Transmigration**—We next investigated the signals connecting ARF6 to PKC. One well-documented ARF effector is phospholipase D (PLD), which catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) (37, 38). Two PLD enzymes have been cloned from mammalian cells, both of which are stimulated by ARF (although PLD1 exhibits more sensitivity to ARF than PLD2 (39)). PLD enzymatic activity can be measured by the incorporation of a primary alcohol in place of water in the phospholipase reaction to create a novel phosphatidylalcohol product. In addition, because the cell cannot recognize or metabolize phosphatidyalcohols as it does PA, primary alcohols also act as PLD inhibitors. Invasion of MDCK cells with wild-type, but not hilA, Salmonella generated a small (1.4-fold) but significant (p = 0.0077) increase in PLD activity (Fig. 6A). In comparison, non-specific activation of the total pool of cellular PLD with the phorbol ester PMA produced a 2.6-fold increase in phosphatidylbutanol, emphasizing that the localized signal elicited by Salmonella is relatively potent.

We then examined whether increased PLD activity during Salmonella invasion was necessary for PMN transmigration. Cells were pretreated with 1-butanol prior to addition of bacteria and PMN to ensure that only epithelial PLD was being inhibited. tert-Butanol, which cannot be used by PLD in the

\(^2\) M. Silva and B. McCormick, unpublished observations.

\(^3\) B. McCormick and J. B. Matthews, unpublished observations.
transphosphatidylation reaction, served as a control for non-specific effects of alcohol. Pretreatment of MDCK monolayers with 1-butanol inhibited PMN transmigration to 45% of control (untreated cells), whereas tert-butanol had no effect (Fig. 6B). Similar results were obtained for PMN transmigration across T84 monolayers. Importantly, 1-butanol had no effect on apical Salmonella entry at this concentration. Therefore, PLD activity, like ARF6 activity, is required for Salmonella-induced PMN transmigration.

To confirm that the bacteria-induced activation of PLD was dependent on ARF6, we expressed an ARF6 construct with a point mutation in its effector loop that renders it unable to activate PLD, without affecting its ability to bind or hydrolyze GTP (N48R (40)). Expression of ARF6(N48R) in MDCK monolayers inhibited Salmonella-induced PMN transmigration to the same extent as the dominant-negative ARF6(T27N) construct (Fig. 6C). This result strongly suggests that the increase in PLD activity in response to apically applied Salmonella is due to activation of ARF6.

To examine which PLD isoform participates in epithelial signaling to PMN, cytosolic and membrane fractions from the PKC translocation experiment in Fig. 4A were immunoblotted with PLD isoform-specific antisera. Under resting conditions, PLD1 was primarily cytosolic, whereas PLD2 was partially detected on membranes (both Triton X-100-soluble and -insoluble membranes). Both PLD1 and PLD2 translocated to a detergent-insoluble membrane compartment, either one or both may participate in epithelial signaling to PMN. Future experiments with mutants of PLD isoforms will pinpoint whether one or both of these enzymes modulate this response.

PKC Translocation Is Dependent upon the Activity of PLD—Having implicated ARF6, PLD, and PKC in Salmonella-induced PMN transmigration across epithelial monolayers, we examined how these three proteins interact with one another to promote this response. Because PKC is an allosteric activator of PLD (37), we initially considered that PKC and ARF6 would synergistically stimulate PLD activity following bacterial invasion. However, treatment with the PKC catalytic site inhibitor

---

**Fig. 6. Requirement for phospholipase D activity during S. typhimurium-induced PMN transmigration.** A, T23 cells prelabeled with [3H]oleic acid were assayed for PLD activity in the absence of bacteria (Control) or in the presence of wild-type (+SL1344) or hilAΔ (inv−) S. typhimurium as described under “Experimental Procedures.” 100 nM PMA was added to the cells as a positive control. Results represent the overall average of duplicates from four separate experiments and are expressed as a-fold induction of PLD activity relative to the uninfected control. *, p < 0.01 (Student’s t test). B, monolayers of T23 cells were pretreated with 0.5% (v/v) 1-butanol or tert-butanol (T-Butanol) in HBSS, or maintained in HBSS alone (Control), prior to addition of SL1344. Transmigrated PMN were quantified as in Fig. 2. **, p < 0.01. C, PMN transmigration was measured across monolayers of T23 cells inductively expressing ARF6(N48R), which cannot activate PLD, or ARF6(T27N). Control, monolayers infected with ARF6(N48R) adenovirus in the presence of 20 ng/ml doxycycline to repress expression. ***, p < 0.025. D, T23 cells apically infected for 60 min (+SL1344) or left uninfected (Control) were lysed and separated into cytosol (C), Triton X-100-soluble membranes (S), and Triton X-100-insoluble membranes (I) as described under “Experimental Procedures.” 150 μg of each fraction was immunoblotted with antisera specific for PLD1 or PLD2.
chelerythrine chloride did not affect PLD activity in the presence of *Salmonella*. We therefore examined the converse possibility that activation of PLD, and the subsequent production of PA, leads to the recruitment of PKC to membranes. To investigate this question, PKC translocation in response to *Salmonella* was measured in the presence of 1-butanol. At a concentration that significantly inhibited PMN transmigration across MDCK monolayers (see Fig. 6B), 1-butanol, but not tert-butanol, prevented PKC redistribution to a membrane fraction (Fig. 7A). A similar result was obtained in the T84 cell model. These results strongly suggest that PLD activity and PA production are required at a stage proximal to PKC translocation.

The recruitment of PKC to membranes is due in part to its association with the lipid second messenger diacylglycerol (DAG). Stimulus-induced DAG production can be achieved either by a phospholipase C activity or by metabolism of PA (derived from PC via PLD) by PA phosphohydrolase (PAP) (37). A similar result was obtained in the T84 cell model. These results strongly suggest that PLD activity and PA production are required at a stage proximal to PKC translocation.

DISCUSSION

Although significant progress has been made in our understanding of *S. typhimurium* internalization by normally non-phagocytic epithelial cells, comparatively little is known about how these pathogens initiate proinflammatory signaling cascades that result in directed PMN transmigration across the intestinal epithelium. In the present study, we provided evidence for the epithelial regulation of PMN transmigration by the GTPase ARF6, which is illustrated in Fig. 8. In this model, *S. typhimurium* contacting the apical surface of polarized epithelial cells elicits a signal, through the bacterial effector SipA, that recruits an ARF6 GEF (such as ARNO) to the plasma membrane. ARNO facilitates ARF6 activation at the apical membrane, which in turn stimulates PLD recruitment to, and activity at, this site. The PLD product PA is metabolized by a phosphohydrolase into DAG, which recruits cytosolic PKC to the apical membrane. Activated PKC phosphorylates downstream targets that are responsible for the production and apical release of PEEC, which drives transepithelial PMN movement. To our knowledge, ARF6 is the first example of a “molecular switch” specifically modulating the PMN transmigration aspect of *S. typhimurium* pathogenesis, independently of bacterial internalization or the release of other proinflammatory mediators such as IL-8.

Although ARF6 was initially identified as a modulator of vesicular traffic and of cortical actin cytoarchitecture, recent evidence suggests that ARF6 is also a regulator of signal transduction cascades initiated by a variety of extracellular stimuli. ARF6 becomes activated by ARNO family exchange factors following insulin stimulation and promotes subsequent glucose transporter translocation to the cell surface and membrane ruffling (20, 21, 42, 43). In ovarian follicular membranes, ARF6 is directly activated following engagement of the luteinizing hormone/chorionic gonadotropin receptor, a heterotrimeric G protein-coupled receptor, and is necessary for β arrestin-mediated receptor down-regulation (22). ARF6 translocates to the plasma membrane of chromaffin cells following nicotine stimulation and, along with PLD, is required for the resulting exocytotic event (44). In phagocytes, FMLP-dependent superoxide production is regulated by ARF6 and PLD (40). Furthermore, ligation of FcγRI promotes formation of a trimeric complex of ARF6, PLD1, and PKCα, which is necessary for activation of other PKC isoforms (45). Although the identity of the cellular “receptor” for *Salmonella* and/or its effector SipA is not known, our observations would suggest that bacterial adhesion to the apical membrane of polarized epithelial cells promotes ARF6 activation in a comparable manner.

We have attempted to directly monitor activation of ARF6 in response to apically invasive *Salmonella* using a recently described co-precipitation assay (30). Although our preliminary results suggest that ARF6 becomes transiently activated during this process, we have so far been unable to conclusively demonstrate this point. Such limited activation is not surprising, because the ARF6 recruited to sites of bacterial interaction with the apical plasma membrane probably represents a small fraction of the total ARF6 pool, as evidenced by the small but significant increase in (ARF6-mediated) PLD activity measured in Fig. 6A.

Recent work has demonstrated that *S. typhimurium* has the ability to directly modulate host GTPase activity through its SPI-1-secreted proteins SopE and SopE2 (46–48). Because the activation of an ARF6-dependent signaling cascade depends...
upon the SPI-1-secreted protein SipA, one possibility is that SipA acts as an ARF6-specific GEF. However, we find this scenario unlikely, because SipA can stimulate PMN transmigration without entering the epithelial cytosol. Instead, we propose that Salmonella/SipA contact with the apical membrane generates a signal that leads to the recruitment of an endogenous ARF GEF. This GEF is most likely of the ARNO family, which includes ARNO, cytosolin-1, GRP1, and ARNO4, because stimuli that lead to ARF6 activation also recruit these GEFs to the plasma membrane. Accordingly, ARNO was detected along with ARF6 at apical sites of Salmonella infection (Fig. 1A). ARF GEF recruitment to the lipid bilayer is primarily mediated by its phosphoinositide-binding pleckstrin homology domain (24). Although ARNO binds with relatively high affinity to both PIP2 and PIP3 (49), pharmacological inhibition of phosphatidylinositol 3-kinase activity was not found to block Salmonella-induced PMN transmigration. We therefore favor a model in which PIP2, an abundant phospholipid in the plasma membrane, is the binding partner for the GEF pleckstrin homology domain. Notably, the resulting activation of PKC may be important for attenuation of the ARF6 signal. PKC phosphorylation of ARNO in its C-terminal polybasic domain has been shown to destabilize the membrane interaction of the GEF with phosphoinositides (49). Because ARNO interacts productively with ARF only at a lipid surface, the cytosolic redistribution of ARNO effectively prevents further activation of ARF.

In the present study we implicate the ARF6 effector phospholipase D (PLD) as a modulator of Salmonella-induced PMN transmigration. Although the product of the PLD reaction, phosphatidic acid (PA), has the ability to alter membrane curvature (37), inhibiting PLD did not affect Salmonella internalization, making it unlikely that PA is required for the membrane ruffling that accompanies bacterial entry. Instead, PA appears to be acting as a lipid second messenger. Proteins, including Raf-1 kinase and ARFs, bind PA selectively (50, 51), and local PA production may help to concentrate ARF6, as well as other potential signal transducing proteins, at sites of bacteria-host cell contact. Interestingly for our current observations, PA has been reported to directly bind and activate PKC in a DAG-independent manner (reviewed in Ref. 52). PA also serves as an activator of another ARF effector with lipid remodeling activity, phosphatidylinositol-4-phosphate 5-kinase (53). PIP2, the product of this reaction, is not only the binding partner for ARF GEFs, like ARNO, but is also a necessary cofactor for activation of PLD (37). Thus, concentrating PA and PIP2 at the apical membrane could bring ARF6, its GEF, and two of its effectors into close proximity.

Previously, we have shown that the kinase activity of PKC is required for basolateral-to-apical PMN transmigration in response to Salmonella and the effector SipA (8). We extend this observation here by demonstrating that activation of ARF6 and PLD downstream of bacteria-epithelial cell interaction is required for the translocation of PKC from the cytosol to membranes, specifically to the apical plasma membrane. Our results suggest that ARF6 and PLD act at a point upstream of PKC activation, because the PKC catalytic site inhibitor chelerythrine chloride did not prevent PLD activation. However, we cannot rule out the possibility that PKC participates in activation of PLD, which occurs independently of PKC kinase activity (37). In this circumstance, bringing together PLD and PKC in the same membrane compartment would facilitate activation of both proteins.

Our preliminary results suggest that PLD promotes the activation of PKC through metabolism of its lipid product, PA, into DAG, a necessary cofactor for activation of classic and novel PKC isoforms. This method of DAG production has been extensively studied in neutrophils, where PA-derived DAG is important for regulation of the oxidative burst (41). Notably, a signaling role for PA-derived DAG remains contentious, because PKC is not as strongly activated by this lipid as by the DAG produced by phospholipase C (which, being derived from PIP2 rather than PC, has a different acyl group composition) (54). However, if PA itself were directing the membrane translocation of PKC, propanolol treatment (which should elevate PA levels while preventing DAG production) would potentiate, not inhibit, PKC translocation. Future experiments analyzing the abundance and molecular composition of DAG during bacterial invasion will conclusively demonstrate whether PAP is involved in PKC recruitment.

The data presented in this report indicate that classic PKC isoforms participate in epithelial cell signaling to PMN. To date, we have not discerned which isoform(s) of PKC regulate the PMN transmigration response. MDCK cells express a subset of PKC isoforms, including the classical isoforms α and β and the novel (responsive to DAG but not Ca2+) isoform δ (55). Interestingly, the PKC inhibitor that was found to block PMN transmigration, chelerythrine chloride (8), acts upon both classical and novel PKC isoforms. In correlation, we also observed membrane translocation of the novel PKC isoforms δ and η in response to Salmonella. Experiments with isoform-specific
PKC inhibitors, as well as activation assays for PKC isoforms, will help to identify the PKC(s) responsible for this event.

What substrates for PKC phosphorylation are required for PMN transmigration? PKC is a known initiator of mitogen-activated protein kinase (MAPK) cascades in many cell types, including MDCK cells, where activation occurs downstream of purinergic receptor activation (56–58). We are currently investigating whether this family of serine/threonine kinases participates in epithelial signaling to PMN. Another target of PKC is the regulatory light chain of myosin II, which in epithelial cells is found in abundance in the actin bundles that attach to junctional complexes. Phosphorylation of myosin light chain by PKCα stimulates the contractile activity of the motor, producing an overall increase in epithelial permeability (59). Although such a mechanism would not account for PEEC synthesis and release, an effect of PKC on epithelial barrier function would assist pathogen-induced PMN movement. Future experiments will focus on identifying proteins phosphorylated by PKC during Salmonella-host cell interaction and should provide a connection between PKC activation and apical release of the novel chemotaxant PEEC.

PMN transmigration across the intestinal epithelium during acute phases of S. typhimurium infection requires the concerted action of multiple proinflammatory factors, including the two PMN chemotaxants IL-8 and PEEC. Recent evidence indicates that different S. typhimurium virulence factors independently promote the release of these cellular products. For instance, the basolateral secretion of IL-8 requires the bacterial product flagellin, through a signaling pathway that utilizes the Ca2+–mediated activation of nuclear factor kappa B (NF-κB) (60, 61). The type III effector protein SopE, which participates in bacterial entry by regulating Rho family GTPase activity, may aid in NF-κB activation (47). Apical release of PEEC is unaffected by this signaling cascade, and instead is dependent upon the SPI-1 bacterial effector protein SipA and, as we have demonstrated here, epithelial ARF6. Although these findings imply that IL-8- and PEEC-directed signaling pathways are independently regulated, these processes are coordinated in vivo such that PMN are mobilized in an effective and rapid manner. By examining potential crosstalk in the signal transduction pathways leading to IL-8 and PEEC release, we hope to gain a better understanding of how these responses direct the PMN movement that characterizes S. typhimurium-induced gastroenteritis.

Acknowledgments—We thank Sylvain Bourgoin, Victor Hsu, Jeff Matthews, Randi Mrsyn, Tom Leto, and Marian Neutra for the reagents and expertise each provided. We also thank Yoram Altschuler and Keith Mostov (University of California at San Francisco) for assistance with production of adenoviruses, Andrew Siber and Isabel Fernandez for help with PMID collection, Samuel Green and Steven Sheridan for critical reading of the manuscript, and members of the Casanova and McCormick laboratories for numerous productive discussions.

REFERENCES
1. Darwin, K. H., and Miller, V. L. (1999) Clin. Microbiol. Rev. 12, 405–428
2. Hueck, C. J. (1998) Microbiol. Mol. Biol. Rev. 62, 379–433
3. McCormick, B. A., Miller, S. I., Carnes, D., and Madara, J. L. (1995) Infect. Immun. 63, 2302–2309
4. McCormick, B. A., Hofman, P. M., Kim, J., Carnes, D. K., Miller, S. L., and Madara, J. L. (1995) J. Cell Biol. 131, 1599–1608
5. McCormick, B. A., Colgan, S. P., Delp-Archer, C., Miller, S. L., and Madara, J. L. (1993) J. Cell Biol. 123, 895–907
6. McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K., and Madara, J. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 274, 12856–12861
7. Mukherjee, K., Parashuraman, S., Mekhada, M. and Mostov, K. (1999) J. Cell Biol. 149, 1144–1154
8.微生物, A. D., Neish, A. S., and Madara, J. L. (2001) J. Cell Biol. 153, 1137–1144