Retinoic Acid-induced Gene-1 (RIG-I) Associates with the Actin Cytoskeleton via Caspase Activation and Recruitment Domain-dependent Interactions*\(^{[5]}\)

Amitava Mukherjee\(^{1}\), Stefanie A. Morosky\(^{1}\), Le Shen\(^{1}\), Christopher R. Weber\(^{4}\), Jerrold R. Turner\(^{4}\), Kwang Sik Kim\(^{4}\), Tianyi Wang\(^{4}\), and Carolyn B. Coyne\(^{11}\)

From the Departments of \(^{1}\)Cell Biology and Physiology and \(^{2}\)Infectious Diseases and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the \(^{3}\)Department of Pathology, The University of Chicago, Chicago, Illinois 60637, and the \(^{4}\)Division of Pediatric Infectious Diseases, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

The actin cytoskeleton serves as a barrier that protects mammalian cells from environmental pathogens such as bacteria, fungi, and viruses. Several components of antimicrobial signaling pathways have been shown to associate directly with the actin cytoskeleton, indicating that the cytoskeleton may also serve as a platform for immune-associated molecules. Here we report that retinoic acid-induced gene-I (RIG-I), an important viral RNA recognition molecule, is associated with the actin cytoskeleton and localizes predominantly to actin-enriched membrane ruffles in non-polarized epithelial cells. Subcellular localization and fractionation experiments revealed that the association between RIG-I and the actin cytoskeleton was mediated by its N-terminal caspase activation and recruitment domains (CARDs), which were necessary and sufficient to induce cytoskeletal association. We also show that RIG-I plays a role in cellular migration, as ectopic expression of RIG-I enhanced cellular migration in a wound healing assay and depletion of endogenous RIG-I significantly reduced wound healing. We further show that in both cultured intestinal epithelial cells (IEC) and human colon and small intestine biopsies, RIG-I is enriched at apico-lateral cell junctions and colocalizes with markers of the tight junction. Depolymerization of the actin cytoskeleton in polarized IEC led to the rapid relocation of RIG-I and to the induction of type I interferon signaling. These data provide evidence that RIG-I is associated with the actin cytoskeleton in non-polarized epithelial cells and with the junctional complex in polarized IECs and human intestine and colon biopsies and may point to a physiological role for RIG-I in the regulation of cellular migration.

The actin cytoskeleton is not only a fundamental component of cellular homeostasis, but in many ways also serves as the first line of host defense against an invading pathogen. Cortical actin filaments directly below the cell membrane form a complex network that provides a barrier to the penetration of viral and bacterial pathogens. This network must therefore be modulated for a pathogen to gain entry into the cell cytoplasm, most commonly via endocytic vesicles. It is therefore not surprising that many pathogens have evolved highly varied strategies to dissolve or modulate the cortical actin meshwork to facilitate cell entry and/or trafficking (1, 2). Thus, the actin cytoskeleton would be ideally suited to act as a platform for immune-associated molecules to sense an invading pathogen and mount an immediate response to promote an antimicrobial state.

Consistent with this, previous studies have shown that several components of the inflammatory pathway interact either directly or indirectly with the actin cytoskeleton. The Gram-positive and -negative bacterial recognition molecule nucleotide oligomerization domain 2 (NOD2)\(^{2}\) associates with the actin cytoskeleton (3) and localizes to cell-cell junctions in intestinal epithelial cells (IECs) (4). Moreover, the proinflammatory caspase caspase-11 directly interacts with the actin interacting protein (Aip1) to promote actin depolymerization mediated by coflin (5) and its activity is regulated by interaction with Flightless-1, a component of actin remodeling (6). The p65 subunit of nuclear factor (NF)-\(\kappa\)B has also been shown to interact with actin filaments, suggesting its activity may also be regulated via this association (7). These findings suggest that components of the inflammatory response tightly associate with the actin cytoskeleton and that this association may regulate inflammatory signal activation.

Disruption of the actin cytoskeleton by the use of actin depolymerizing agents (such as cytochalasin D (cytoD)) activates NF\(\kappa\)B signaling in monocytes (8), human embryonic kidney (3), and polarized intestinal epithelial cells (9). Activation of NF\(\kappa\)B often correlates with the induction of an inflammatory state, indicating that inflammatory signals are generated in response to dramatic alterations in actin cytoskeletal architecture. Taken together, these findings support a model of inflammatory sig-

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\(^{[5]}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

\(^{[1]}\) From whom correspondence should be addressed: S313 Biomedical Science Tower, 3500 Terrace St., Pittsburgh, PA 15261. Tel.: 412-383-5149; Fax: 412-383-6517; E-mail: coyne2@pitt.edu.
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...naling in response to perturbations of the actin cytoskeleton, possibly due to the activation of actin-associated inflammatory components.

The innate immune system responds to essential functional components of microorganisms, which are thus broadly expressed within classes of pathogens (10). Two functionally related intracellular viral recognition molecules recognize cytoplasmic double-stranded RNA that is produced as a replication intermediate in the life cycle of many RNA viruses. Retinoic acid-induced gene-I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) binding to double-stranded RNA initiates signaling events resulting in translocation of interferon (IFN) regulatory factors (IRF)-3 and -7 into the nucleus and subsequent induction of type I IFNs, a key component of antiviral host defense (11, 12). RIG-I and MDA5 each contain two N-terminal caspase activation and recruitment domains (CARDs) and a C-terminal DEXD/H-box RNA helicase domain. The C-terminal domain serves as a regulatory repressor domain that masks the exposure of the CARDs to prevent downstream signaling in the absence of stimulus. Upon RNA binding, structural changes release this domain leading to exposure of the CARD domains and in the induction of downstream antiviral signals.

It remains unclear if either RIG-I or MDA5 serve functions beyond that of the antimicrobial response. Deletion of RIG-I in mice leads to the development of a colitis-like phenotype characterized by severe inflammation of the colon mucosa (13) (in another study, RIG-I-deficient mice die in utero (14)). RIG-I has also been implicated as a negative regulator of granulocytic differentiation in mice (15). These studies would suggest that RIG-I may serve an essential role beyond that of viral RNA recognition and may actively participate in some aspect of development and/or mucosal signaling. However, the precise nature of this role remains undefined.

In this study, we show that RIG-I is concentrated at sites of actin-rich membrane ruffles in non-polarized and polarized epithelial cells and plays a role in cell migration. We further show that RIG-I is enriched at apico-lateral cell junctions of both cultured IECs and human colon biopsies where it colocalizes with markers of the apical tight junction (TJ) complex. The localization of RIG-I to lamellipodia and its association with the actin cytoskeleton is mediated by CARD-dependent interactions, as these domains are both necessary and sufficient to promote membrane ruffle association. Our results show that in addition to serving an important role in innate immune signaling, RIG-I is closely associated with the actin cytoskeleton and may participate in the regulation of cellular motility and migration.

EXPERIMENTAL PROCEDURES

Cells—Caco-2 cells were cultured as described (16, 17). HEK293 and human osteosarcoma U2OS cells were cultured in Dulbecco’s modified Eagle’s medium-high glucose supplemented with 10% fetal bovine serum and penicillin/streptomycin. For all studies, cells were plated in collagen-coated 8-well culture slides (Nunc) or collagen-coated culture dishes. All cells were grown for a minimum of 24 (HEK293, U2OS) or 48 (Caco-2) h prior to study. Cells were screened for mycoplasma using a PCR-based mycoplasma test (Takara Bio USA, Madison, WI) to prevent abnormalities in cellular morphology, transfection, and growth.

Plasmids, siRNAs, and Transfections—All cDNAs were purchased from Origene Technologies (Rockville, MD). The complete open reading frame of RIG-I was isolated by PCR and cloned into the EGFP-C2 expression vectors (Clontech, Mountain View, CA) by insertion into XhoI and HindIII sites. EGFP-RIG-I-CARDS and EGFP-RIG-I-ΔCARDs constructs were constructed similarly. All constructs were verified by sequencing. A pool of four siRNAs (ON-TARGETplus SMARTpool®) against RIG-I was purchased from Dharmacon (Lafayette, CO).

Transfection of Caco-2 cells with plasmids was performed using the Nucleofector System (Amaxa, Gaithersburg, MD) according to the manufacturer’s protocol. Plasmid transfections of HEK293 and U2OS cells were performed using FuGENE 6 according to the manufacturer’s protocol (Roche Applied Science). siRNA transfections were performed with HiPerFect transfection reagent (Qiagen, Valencia, CA). Following transfection, cells were plated as described above and cultured for 24–48 h later. Transfection efficiencies with plasmids (as determined by EGFP expression) were 75–90 (Caco-2), 90–100 (HEK293), and 75–100% (U2OS) and transfection with Cy5-labeled siRNA was 95–100% (HEK293).

Antibodies—Monoclonal antibody against ZO-1 was purchased from Invitrogen. Goat polyclonal antibodies specific for RIG-I (C-15 and L-15), and MDA5 (C-16) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal and mouse monoclonal antibodies directed against GFP (FL, B-2), glyceraldehyde-3-phosphate dehydrogenase (sc-25778), vimentin (sc-73258), and IRF3 (FL-425) were purchased from Santa Cruz Biotechnology. Calpain-2 mouse monoclonal antibody was purchased from Abcam (Cambridge, MA). Alexa Fluor-conjugated secondary antibodies were purchased from Invitrogen. Endogenous RIG-I staining was confirmed with two goat polyclonal antibodies targeting unique epitopes of RIG-I: either the C terminus (C-15) or an internal region (L-15) not containing the CARDs (supplemental Fig. 1A). To confirm the specificity of these antibodies, we performed Western blot analysis of overexpressed EGFP-fused RIG-I full-length, ΔCARDs, or CARDs. As expected, both antibodies recognized full-length and ΔCARDs RIG-I but were incapable of recognizing only the CARDs of RIG-I (which does not contain the epitope for either antibody) (supplemental Fig. 1A).

Immunofluorescence Microscopy— Cultures were washed and fixed with either 4% paraformaldehyde or with ice-cold methanol/acetone (3:1). Cells were then permeabilized with 0.1% Triton X-100 in phosphate-buffered saline and incubated with the indicated primary antibodies for 1 h at room temperature. Following washing, cells were incubated with secondary antibodies for 30 min at room temperature, washed, and mounted with Vectashield (Vector Laboratories, Burlingame, CA) containing 4’,6-diamidino-2-phenylindole. Images were captured with an inverted IX51 Olympus microscope equipped with a cooled monochrome DP30 CCD camera, and controlled by Olympus Slidebook 4.2 advanced imaging software (Olympus, Melville, NY) or a confocal laser-scanning microscope (Leica, Exton, PA). The scale bar in the figures is 10 μm. Images...
were processed using Adobe Photoshop CS3 (Adobe, San Jose, CA).

**Immunohistochemical Staining**—Formalin-fixed, paraffin-embedded small intestine and colon biopsies were obtained under a protocol approved by The University of Chicago Institutional Review Board. Hematoxylin and eosin-stained sections were reviewed by a surgical pathologist and only biopsies deemed normal were used in this study. Adjacent, unstained sections were deparaffinized, rehydrated, and washed. Antigen retrieval was carried out by heating sections in citrate buffer (pH 6) in a microwave oven. After quenching of endogenous peroxidase by incubation with 3% H2O2 in methanol, nonspecific binding sites were blocked by incubation in 5% nonfat milk and tissue sections were incubated with goat anti-RIG-I antibody (C-15, 4 μg/ml) in phosphate-buffered saline with 0.1% Triton X-100 and 0.2% bovine serum albumin. After washing, sections were incubated with horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (R&D Microsystems), washed, developed with 3,3’-diaminobenzidine chromogen, counterstained with hematoxylin, and mounted.

**Live Cell Imaging**—Live cell imaging was performed on Caco-2 cells transfected with EGFP-RIG-I and grown on collagen-coated glass bottom 35-mm dishes (Wilco Imaging, San Diego, CA). Dishes containing transfected cells were transferred to a heated and CO2-controlled DH-35i microincubation system (Warner Instruments, Hamden, CT) positioned over an inverted IX51 Olympus microscope equipped with a cooled monochrome DP30 CCD camera, and controlled by Olympus Slidebook 4.2 advanced imaging software (Olympus). Images were captured using an Olympus UPlanApo 0.85 NA ×40 objective. Images were captured every 10 s prior to and following the addition of cytochalasin D (8 μM) (Calbiochem, San Diego, CA) for a total of 30 min.

**Reporter Gene Assays**—Activation of IFNβ or NFκB promoters were measured by reporter assay. Caco-2 cells were transfected in 24-well plates (as described above) with p-125 Luc (IFNβ) or NFκB reporter plasmid together with the indicated plasmids. Luciferase activity was measured by the Dual Luciferase system (Warner Instruments, Hamden, CT) positioned over an inverted IX51 Olympus microscope equipped with a cooled monochrome DP30 CCD camera, and controlled by Olympus Slidebook 4.2 advanced imaging software (Olympus). Luciferase activity was normalized to that of Renilla, and relative activation was determined. All experiments were performed in triplicate and conducted a minimum of three times.

**Cellular Fractionation**—Caco-2, HEK293, or U2OS cells were cultured in collagen-coated 100-mm tissue culture dishes and subjected to subcellular fractionation using the ProteoEx-tract Subcellular Proteome Extraction kit (Calbiochem, San Diego, CA) according to the manufacturer’s instructions. Cytosolic, membrane/organelle, nuclear, and cytoskeletal fractions were separated using SDS-PAGE followed by immunoblotting as described below.

**Immunoblot Analysis**—Cell lysates were prepared with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 μM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 μM sodium orthovanadate), and insoluble material was cleared by centrifugation for 5 min at 4 °C. Lysates (30–50 mg) were loaded onto 4–20% Tris-HCl gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight in 5% nonfat dry milk or 3% bovine serum albumin, probed with the indicated antibodies, and developed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), and SuperSignal West Pico or West Dura chemiluminescent substrates (Pierce Biotechnology).

**In Vitro Wound Healing Assay**—Assays were performed essentially as described (18). Briefly, HEK293 cells transfected with EGFP-RIG-I, EGFP-CARDs, EGFP-ΔCARD, or EGFP-C2 vectors or with control or RIG-I siRNAs were seeded for 36 h on collagen-coated 12-well culture dishes. Wounds were induced by scratching the cell monolayer with a sterile 200-μl pipette tip. Images were captured by phase-contrast microscopy immediately following wound induction (0 h), and at 12 and 24 h post-scratching. A single wound was induced per experiment. To quantify cellular migration across the width of the wound, the length of 40 individual lines (in μm) throughout the wound were measured at each time point (from three individual wells per experiment). Data are presented as the percent of wound closure from 0 h (time of wound induction) to 12 and 24 h post-wounding.

**Statistical Analysis**—Data are presented as mean ± S.D. A one-way analysis of variance and Bonferroni’s correction for multiple comparisons were used to determine statistical significance (p < 0.05). Data are representative of experiments performed at least three times.

**RESULTS**

**RIG-I Localizes to Membrane Ruffles in Non-polarized Epithelial Cells**—RIG-I has been described as a cytoplasmic sensor of viral infections (11). However, few studies have investigated the precise cellular localization of RIG-I. To determine the subcellular distribution of endogenous RIG-I in non-polarized epithelia, we performed immunofluorescence microscopy in human embryonic kidney HEK293 and human osteosarcoma U2OS cells. We found that in both HEK293 and U2OS cells, endogenous RIG-I localized extensively to membrane ruffles where it colocalized with F-actin (Fig. 1, A and B). In contrast, the RIG-I-related molecule MDA5 was strictly localized to the cytoplasm and exhibited no significant colocalization with F-actin (supplemental Fig. S2A). (This staining was specific for MDA5 as transfection of cells with MDA5 siRNA significantly reduced the level of MDA5 detected by immunofluorescence (not shown).) RIG-I also localized to areas of actin-rich membrane extensions of other non-polarized cells types such as mouse embryonic fibroblasts and HeLa cells (not shown). Endogenous RIG-I staining was confirmed with two goat polyclonal antibodies targeting unique epitopes of RIG-I: either the C terminus (C-15) or an internal region (L-15) not containing the CARDs (for detailed characterization, see supplemental Fig. S1A).

To further analyze the subcellular distribution of RIG-I, we performed cell fractionation based on detergent extraction. Immunoblot analysis of cellular fractions revealed that endogenous RIG-I was present in both the cytosolic and detergent-insoluble cytoskeletal fractions (Fig. 1C), although the propor-
Membrane ruffles (or lamellipodia) are composed of a network of branched actin filaments and are localized to the mobile end of cells. The Rho family of small GTPases (Rho, Rac, and Cdc42) are pivotal components of actin cytoskeletal regulation and when activated, signal to a variety of downstream effector molecules that govern cellular movements. In particular, Rac mediates the formation of focal complex structures associated with lamellipodia/membrane ruffles. RIG-I localization to membrane ruffles was dependent on Rac GTPase activity, as treatment of cells with a specific pharmacological inhibitor of Rac (NSC23766) inhibited the formation of ruffles and led to the relocalization of RIG-I to the cell periphery (Fig. 2A). In contrast, when cells were stimulated with phorbol 12-myristate 13-acetate to induce the formation of ruffles, RIG-I concentration in lamellipodia was enhanced (Fig. 2B).

To confirm the specificity of the association between RIG-I and membrane ruffles, we constructed RIG-I fused to EGFP at its N terminus. We found that overexpressed EGFP-RIG-I also displayed enhanced localization to membrane ruffles in HEK293 cells (Fig. 1D), which was abolished upon treatment of cells with NSC23766 (not shown). Similar results were obtained in U2OS cells (supplemental Fig. S2B). In contrast, an EGFP control exhibited no association with membrane ruffles (Fig. 3B, top row). In addition to the association between EGFP-RIG-I and membrane ruffles, we also observed extensive colocalization between overexpressed EGFP-RIG-I and actin filaments directly below the cells surface (Fig. 1E, top). Similar to our findings with endogenous RIG-I, we found that overexpressed EGFP-RIG-I also fractionated in detergent-insoluble cytoskeletal fractions, with additional accumulation in both the cytosolic and membrane/organellar fractions (Fig. 1F).

The CARDs of RIG-I Are Necessary and Sufficient to Promote Lamellipodia Localization—RIG-I contains two N-terminal CARDs and a C-terminal DEHD/H-box RNA helicase domain. Many pro-apoptotic and pro-inflammatory molecules contain CARDs, which serve to not only mediate specific interactions that promote signaling, but also promote homotypic and heterotypic protein interactions. The CARDs of RIG-I are involved in homophilic CARD-CARD interactions with a downstream adaptor molecule, mitochondrial antiviral signaling (also known as VISA/IPS-1/Cardif) that activates NFκB and IRF3 to...
regulate type I interferon production (19–22). The CARDs of other CARD-containing proteins have been shown to mediate actin cytoskeletal association, both NOD2 and caspase-11 interact with components of the actin cytoskeleton through CARD interactions (3, 5). To determine which domain of RIG-I was involved in its association with the actin cytoskeleton, we constructed EGFP-tagged constructs of RIG-I lacking both CARDs (RIG-I/CARDs) or containing the CARDs alone (RIG-I CARDs) and determined their extent of cytoskeletal association by subcellular localization and fractionation studies (Fig. 3A).

Confocal microscopy studies revealed that whereas ectopically expressed EGFP-RIG-I localized extensively to membrane ruffles in U2OS cells, control EGFP did not exhibit significant ruffle association (Fig. 3B). In contrast to full-length EGFP-RIG-I, we found that EGFP-CARDs exhibited no ruffle association, indicating that the CARDs of RIG-I are necessary to confer actin cytoskeletal association (Fig. 3B). Consistent with this, we found that EGFP-CARDs exhibited extensively lamellipodia association (Fig. 3B). We also found that Discosoma sp. red fluorescent protein-tagged CARDs colocalized with EGFP-RIG-I to sites of membrane ruffles when coexpressed in HEK293 cells (supplemental Fig. S2C).

In biochemical fractionation experiments performed in HEK293 cells transfected with EGFP-tagged RIG-I constructs, we found that EGFP-CARDs did not fractionate in the cytoskeletal fraction and instead resided specifically in the cytosolic and to some extent membrane/organelle fractions (Fig. 3C). In contrast, both full-length EGFP-RIG-I and EGFP-CARDs associated heavily with the cytoskeletal fraction (Fig. 3C). These results show that the CARDs of RIG-I are both necessary and sufficient to confer cytoskeletal association.

RIG-I Plays a Role in Cellular Migration—The pronounced association between RIG-I and actin-enriched membrane ruffles led us to investigate whether RIG-I may play a role in the regulation of cell migration, a process that involves both the polymerization and depolymerization of actin filaments in lamellipodia. The in vitro wound healing assay is one of the earliest and most widely used means by which to measure cell migration and is based upon the healing of wounds in vivo (18). Using this method, we found that overexpression of EGFP-RIG-I led to an enhancement of cellular migration in HEK293 cells (Fig. 4A). Concomitantly, inhibition of RIG-I expression by siRNA transfection led to a modest decrease in cellular migration (Fig. 4B).

Because we found that deletion of the CARDs of RIG-I abolished ruffle association (Fig. 3, B and C), we determined if the CARDs of RIG-I were involved in the enhancement of cellular migration upon EGFP-RIG-I overexpression. To do this, we expressed EGFP-CARDs and determined the effects of this overexpression on cellular migration in a wound healing assay. In contrast to our findings with ectopic EGFP-RIG-I expression, we found that overexpression of EGFP-CARDs did not
enhance cellular migration (Fig. 4A), indicating that the CARDs or RIG-I are necessary to induce cellular migration.

**RIG-I Is Associated with Membrane Ruffles in Non-confluent Caco-2 Cells**—The CARD-containing bacterial recognition receptor NOD2 has been shown to localize membrane ruffles in HEK293 cells (3) and to cellular junctions in polarized intestinal epithelial cells (4). Our data indicate that RIG-I shares a striking pattern of membrane ruffle localization with NOD2 in HEK293 cells. Because of this, we determined the localization of RIG-I in monolayers of Caco-2 cells, a cell line derived from a human colorectal carcinoma that differentiates under standard culture conditions. We found that in subconfluent Caco-2 cells, RIG-I was predominantly associated with F-actin at membrane ruffles along the cell periphery and exhibited no association with other actin-rich structures (such as stress fibers or filopodia (Fig. 5A)). Additionally, overexpressed EGFP-RIG-I also exhibited association with membrane ruffles of non-confluent Caco-2 cells (Fig. 7F).

**RIG-I Associates with the Apical Junction Complex of IECs**—In confluent Caco-2 cells, we found that RIG-I was closely associated with the apical tight junction (TJ) complex where it colocalized strongly with ZO-1, a marker of the TJ (Fig. 5B). In contrast, MDA5 was restricted to the cytoplasm (Fig. 5C). Many components of the actin cytoskeleton have been localized to apical TJs in confluent polarized epithelial cells (23, 24). However, in subconfluent monolayers, these proteins often associate specifically with actin-associated structures such as stress fibers or filopodia (25). Junctional association of RIG-I was confirmed in a number of other gastrointestinal tract-derived cell lines including human intestinal T84 cells and colon-derived HT-29 (not shown). However, the association between RIG-I and TJs was strongest in both parental Caco-2 and Caco-2 brush-border epithelium cell lines.
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As polarized endothelial monolayers also form apical TJs, we investigated the association of RIG-I with TJs in polarized human brain microvascular endothelial cells, a model of the blood-brain barrier (17). We found that in human brain microvascular endothelial cells, RIG-I was extensively associated with F-actin-enriched membrane ruffles in subconfluent human brain microvascular endothelial cells and with the junctional complex in confluent cultures (supplemental Fig. S3). These data indicate that the localization of RIG-I to cellular junctions is not specific for polarized IECs and that it also is enriched at cellular junctions of polarized endothelial cells.

To confirm the association between RIG-I and the actin cytoskeleton in Caco-2 cells, we performed cell fractionation based on detergent extraction. Immunoblot analysis of cellular fractions revealed that endogenous RIG-I was present only in the detergent-insoluble cytoskeletal fraction (Fig. 5D). (In contrast, in HEK293 cells, endogenous RIG-I also was present in the cytosolic fraction (Fig. 1O).)

To confirm the association between RIG-I and TJs, we analyzed the distribution of EGFP-RIG-I in polarized Caco-2 cells. Similar to our findings with endogenous RIG-I, we found that overexpressed EGFP-RIG-I associated with the apical junctional complex of Caco-2 cells (Fig. 5E).

RIG-I Is Associated with the Junctional Complex in Human Colonic and Intestinal Epithelium Biopsies—We observed extensive association between RIG-I and the apical junctional complex of Caco-2 cells (Fig. 5B). However, as Caco-2 cells are an immortalized colon cancer cell line that may exhibit morphological abnormalities, we investigated the localization of RIG-I in sections isolated from normal human colon and small intestine biopsy sections. In human sections of surface colonic, villus enterocytes, and small intestine epithelium, RIG-I exhibited strong association with the TJ complex and also significant association with the apical membrane (which is likely indicative of its association with the cortical actin ring and microvillus actin network (Fig. 6)).Taken together, these results clearly show that RIG-I associates with TJs and the actin cytoskeleton in an immortalized human intestinal cell line, Caco-2 cells (Fig. 5B), and in human colonic and intestinal epithelium (Fig. 6).

Actin Depolymerization Stimulates RIG-I Relocalization—Because we observed a close association between RIG-I and the actin cytoskeleton in polarized IECs, we investigated the effect of actin depolymerization on RIG-I localization. We found that cytoD-mediated actin depolymerization led to the relocalization of RIG-I from the junctional complex to actin-enriched clusters (which may represent either or both cytoplasmic vesicles or residual F-actin from the cortical ring (Fig. 7A)). These structures did not colocalize with ZO-1, a component of the TJ, but were extensively associated with actin (Fig. 7A). In contrast, MDA5 localization was unaffected by actin depolymerization (not shown).

Actin Cytoskeleton Disruption Activates Type I IFN Signaling in IECs—Because our studies showed extensive relocalization of RIG-I in response to actin depolymerization, we investigated the effect of actin cytoskeleton disruption on NFκB and IRF3 activation and type I IFN signaling. RIG-I activation (in response to double-stranded RNA binding) initiates signaling events resulting in the translocation of IRF3 and IRF7 to the nucleus and to the subsequent induction of type I IFN transcription (11, 12). Although previous studies have reported NFκB activation in response to cytoD treatment of IECs (9), the effect of this treatment on IRF3 localization and type I IFN signaling is unclear.

We found that exposure of Caco-2 cells to cytoD led to the pronounced activation of IRF3 (as evidenced by its nuclear translocation (Fig. 7C)). Exposure of cells to cytoD also induced the robust activation of NFκB and IFNβ promoter activity as assessed by transfection of cells with IFNβ- and NFκB-luciferase constructs (Fig. 7B), indicating that the induction of type I IFNs is activated downstream of actin reorganization.

The degree of polarization of Caco-2 cells appeared to have no impact on either the relocalization of RIG-I or the induction of IRF3 signaling as cytoD treatment of subconfluent Caco-2 cells induced the relocalization of RIG-I to actin clusters (Fig. 7D) and led to IRF3 nuclear translocation (Fig. 7E).

Real-time Relocalization of EGFP-RIG-I in Response to Actin Depolymerization—In subconfluent Caco-2 cultures, EGFP-RIG-I localized to membrane ruffles, consistent with our findings with endogenous RIG-I (Figs. 5A and 7F). We used time-lapse imaging to determine the kinetics of EGFP-RIG-I relocalization in response to cytoD treatment of subconfluent Caco-2 cells. These studies revealed the rapid relocalization of EGFP-RIG-I from actin-rich membrane ruffles to punctate domains (which are likely heavily associated with actin) within 5 min of cytoD exposure (Figs. 7B and supplemental S4). This relocalization corresponded with morphological changes that included the collapse of membrane ruffles and cortical actin meshwork as a consequence of cytoD treatment.
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**DISCUSSION**

RIG-I is a key regulator of the type I IFN antiviral signaling pathway. Here we show by confocal microscopy and subcellular fractionation studies that endogenous and ectopically expressed RIG-I is associated with the actin cytoskeleton and is primarily expressed at sites of actin-rich membrane ruffles in non-polarized epithelial cells. The CARDs of RIG-I were necessary and sufficient to mediate lamellipodia association as ectopically expressed RIG-I lacking both CARDs was incapable of cytoskeletal association and expression of the CARDs alone conferred lamellipodia localization and cytoskeletal association. We further show in a wound healing assay that RIG-I regulates cellular migration, indicating that its localization to the cytoskeleton may serve a physiological function. Our results also show that RIG-I is enriched at apico-lateral cell junctions of both polarized IECs and human colon and intestine biopsies and is relocalized by depolymerization of the actin cytoskeleton. The induction of IRF3-mediated type I IFN signaling in response to cytoD treatment may indicate that release of RIG-I from the cytoskeleton leads to the induction of antiviral signaling. Taken together, these data may highlight a novel role for RIG-I in actin cytoskeleton regulation and suggest that its localization within the cell may play an important role in innate immune signaling.

The actin cytoskeleton is commonly modulated by invading viruses as a means to facilitate cellular uptake and/or trafficking. Because this modulation often occurs very early in the virus lifecycle (generally well before the release of genomic content and replication), the actin cytoskeleton would be ideally suited to act as a platform for innate immune-associated molecules to sense an invading pathogen and mount an immediate antimicrobial response. The disassembly of the actin cytoskeleton associated with microbial entry may thereby release actin-associated innate immune molecules from their cytoskeletal/junctional associations and allow for the induction of downstream signaling. Although it is unclear whether this form of activation exists for RIG-I and other innate immune molecules, given the growing numbers of associations between various components of innate immune and inflammatory signaling with the actin cytoskeleton, it would seem to be a plausible strategy utilized by the host cells to mount an almost immediate antimicrobial response to the invading pathogen. Coupled with a secondary response that would depend on the recognition of viral genome content or RNA replication intermediates, the cytokine signaling may play an important role in innate immune signaling.

Our results show that depolymerization of the actin cytoskeleton leads to the pronounced activation of NFκB and type I IFN signaling pathways. Specifically, we observed extensive relocalization of the type I IFN inducer IRF3 to the nucleus in polarized IECs following exposure to cytoD. This relocalization was specific for polarized IECs, as IRF3 was not relocalized to the nucleus in cytoD-treated HEK293 cells. This relocalization to the nucleus in cytoD-treated HEK293 cells (not shown). It is unclear which innate immune-associated molecules may function upstream of this activation. Although NOD2 is expressed at low levels in Caco-2 cells (26) and may function upstream of NFκB activation response to actin depolymerization (as has
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been shown in HEK293 cells (31), NOD2 does not activate IRF3 (27), which may support a role for RIG-I in this response.

Although the mechanisms by which the innate immune system recognizes foreign pathogens to mount an antimicrobial response have been studied extensively, there is much to be learned about how these pathways function in IECs. Given that IECs contain specialized membrane domains and are tightly regulated by modulation of either the actin cytoskeleton or junctional complex, studies on the cell biological properties of innate immune-associated molecules may yield important clues as to the regulation of mucosal immunity. Studies on the localization of Toll-like receptors, important regulators of antiviral and antibacterial signaling, in polarized airway cells and IECs have revealed the asymmetric distribution of Toll-like receptors to the apical and basolateral membrane domains (28).

Because the intestinal epithelium is in constant contact with commensal bacteria within the lumen, this polarity of distribution is likely a fundamental aspect in the maintenance of intestinal homeostasis. Although information regarding the role of Toll-like receptors in maintaining IEC homeostasis and microbial recognition is expanding, little is known regarding the mechanisms by which RIG-I signaling is regulated in these cells or whether its cellular localization may contribute to its function in antiviral signaling. Our results show that RIG-I is closely associated with the actin cytoskeleton in and localizes predominantly to the junctional complex in polarized IECs. It is well established that the actin cytoskeleton plays a critical role in maintaining TJ architecture and that even subtle perturbation of the actin cytoskeleton lead to drastic loss of TJ function and to the relocation of TJ-associated components (29). Given the growing number of virus receptors that have been shown to associate with the junctional complex of polarized epithelia (30–35), the localization of RIG-I to the apical junction complex would serve not only to bring it within close proximity to a number of viruses that would likely gain entry through this complex, but would localize it to a structure within the cell that is tightly regulated by any modification of the actin cytoskeletal architecture.

RIG-I acts as an important sensor for the detection of viral infection and initiates potent antiviral signals to combat these infections. Here we report that in addition to its central function in viral innate immune recognition, RIG-I plays a role in actin cytoskeletal regulation and localizes specifically to membrane ruffles in non-polarized epithelial cells and to the junctional complex of polarized IECs and human colon biopsies. It is conceivable that the association between RIG-I and the actin cytoskeleton may be related to fundamental mechanisms involved in RIG-I-mediated innate immune recognition, particularly in the polarized IECs of the gastrointestinal tract.

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