Mechanism of IFN- γ -induced Endocytosis of Tight Junction Proteins: Myosin II-dependent Vacuolarization of the Apical Plasma Membrane

Markus Utech,^{*†} Andrei I. Ivanov,^{*‡} Stanislav N. Samarin,^{*‡} Matthias Bruewer,[†] Jerrold R. Turner,[§] Randall J. Mrsny,^{||} Charles A. Parkos,^{*} and Asma Nusrat^{*}

^{*}Epithelial Pathobiology Research Unit, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA 30322; [†]Department of General Surgery, University of Muenster, 48149 Muenster, Germany; [‡]Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 142290 Pushchino, Russia; [§]Department of Pathology, The University of Chicago, Chicago, IL 60637; and ^{||}Welsh School of Pharmacy, University of Wales, Cardiff CF10 3XF, Wales, United Kingdom

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Disruption of epithelial barrier by proinflammatory cytokines such as IFN- γ represents a major pathophysiological consequence of intestinal inflammation. We have previously shown that IFN- γ increases paracellular permeability in model T84 epithelial cells by inducing endocytosis of tight junction (TJ) proteins occludin, JAM-A, and claudin-1. The present study was designed to dissect mechanisms of IFN- γ -induced endocytosis of epithelial TJ proteins. IFN- γ treatment of T84 cells resulted in internalization of TJ proteins into large actin-coated vacuoles that originated from the apical plasma membrane and resembled the vacuolar apical compartment (VAC) previously observed in epithelial cells that lose cell polarity. The IFN- γ dependent formation of VACs required ATPase activity of a myosin II motor but was not dependent on rapid turnover of F-actin. In addition, activated myosin II was observed to colocalize with VACs after IFN- γ exposure. Pharmacological analyses revealed that formation of VACs and endocytosis of TJ proteins was mediated by Rho-associated kinase (ROCK) but not myosin light chain kinase (MLCK). Furthermore, IFN- γ treatment resulted in activation of Rho GTPase and induced expressional up-regulation of ROCK. These results, for the first time, suggest that IFN- γ induces endocytosis of epithelial TJ proteins via RhoA/ROCK-mediated, myosin II-dependent formation of VACs.

INTRODUCTION

Disruption of epithelial barriers represents a hallmark of systemic and local inflammation and it may be caused by either bacterial and viral pathogens or by endogenous mediators such as proinflammatory cytokines (Karlinger *et al.*, 2000; Sartor, 2003). Increased mucosal permeability is thought to be an early and critical step in pathogenesis of Crohn's disease and ulcerative colitis, collectively known as inflammatory bowel disease (IBD). One of the potential molecular triggers of intestinal barrier disruption in IBD patients is interferon (IFN) γ . Indeed, expressional up-regulation of this cytokine is well documented in mucosal biopsies of IBD patients (Niessner and Volk, 1995; Stallmach *et al.*, 2004). IFN- γ signals through its receptor on the cell surface leading to transcriptional activation of different target genes.

Such signaling events induce a variety of cellular responses including inhibition of ion secretion (Colgan *et al.*, 1994), activation of the cAMP signaling pathways (Kolachala *et al.*, 2005), and apoptosis (Ruemmele *et al.*, 1999; O'Connell *et al.*, 2000). A prominent effect of IFN- γ on model intestinal epithelial monolayers is an increase in paracellular permeability and disruption of the epithelial barrier (Madara and Stafford, 1989; Bruewer *et al.*, 2003). We recently demonstrated that such barrier disruption occurs independent of enterocytes apoptosis (Bruewer *et al.*, 2003). However, molecular mechanisms of IFN- γ induced increase in epithelial permeability remain poorly understood.

Barrier properties of polarized epithelial monolayers are primarily determined by a multiprotein complex located at the most apical part of the lateral plasma membrane referred to as the tight junction (TJ). TJs consist of transmembrane and cytosolic components (Tsukita *et al.*, 2001; Gonzalez-Mariscal *et al.*, 2003). The former interact with their counterparts on the opposing plasma membrane thus sealing the intercellular space, whereas the latter link transmembrane components of TJs to the underlying F-actin bundles and also play a role in intracellular signaling. The major transmembrane proteins of the TJ include occludin, members of the claudin family, and two immunoglobulin-like proteins, junctional adhesion molecule (JAM)-A and coxsackie adenovirus receptor. Intracellular components of TJs have been shown to create a so called cytosolic plaque that includes over 30 different proteins. Among them, the most studied are three members of the *zonula occludens* (ZO) protein fam-

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Address correspondence to: Asma Nusrat (anusrat@emory.edu).

Abbreviations used: Arp 3, actin-related protein 3; IFN- γ , interferon- γ ; JAM, junctional adhesion molecule; MLC, myosin light chain; MLCK, myosin light chain kinase; MNMM, mammalian nonmuscle myosin; MYPT, myosin phosphatase target subunit; PFA, paraformaldehyde; ROCK, Rho-associated kinase; TJ, tight junction; VAC, vacuolar apical compartment; VASP, vasodilator-stimulated phosphoprotein.

ily (ZO-1, ZO-2, and ZO-3) that bind to TJ transmembrane proteins and the underlying perijunctional F-actin ring (Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Gonzalez-Mariscal *et al.*, 2003).

Several recent studies demonstrated that the increase in mucosal permeability in IBD patients is accompanied by disruption of normal morphology of TJs in the intestinal epithelial lining (Schmitz *et al.*, 1999; Kucharzik *et al.*, 2001). Furthermore, exposure of intestinal epithelial monolayers to proinflammatory cytokines, IFN- γ and TNF- α was associated with disassembly of TJs and increased paracellular permeability across epithelial cells (Bruewer *et al.*, 2003). Recently we demonstrated that IFN- γ disrupts barrier function in model T84 intestinal epithelial cells by inducing selective internalization of TJ transmembrane proteins but not its cytosolic plaque components (Bruewer *et al.*, 2003). TJ proteins were internalized by macropinocytosis and temporarily stored in a recycling endosomal compartment from where they were observed to be recycled back to the plasma membrane after withdrawal of IFN- γ (Bruewer *et al.*, 2005). In mucosal biopsies from patients with actively inflamed ulcerative colitis, substantial fractions of TJ transmembrane proteins were observed in subapical vesiclelike structures, supporting our *in vitro* observations (Bruewer *et al.*, 2005).

The mechanism by which IFN- γ triggers macropinocytosis of epithelial TJ proteins remains enigmatic. One of the characteristic features of macropinocytosis is its dependence on reorganization of cortical actin cytoskeleton. Indeed, the formation of macropinosomes starts with actin-dependent membrane ruffling that eventuates in the creation of large F-actin-coated vacuoles (Swanson and Watts, 1995; Amyere *et al.*, 2002). On the other hand, TJs have been previously shown to be directly linked to thick F-actin bundles that are crucial for junctional assembly and integrity (Madara, 1987; Stevenson and Begg, 1994; Fanning *et al.*, 1998). Furthermore, our recent findings suggested that reorganization of perijunctional F-actin drives disassembly and internalization of apical junctions in calcium-depleted epithelial cells (Ivanov *et al.*, 2004a). On the basis of these data we hypothesized that IFN- γ triggers macropinocytosis of epithelial TJ proteins by inducing reorganization of the actin cytoskeleton. The present study was designed to test this hypothesis and to elucidate mechanisms of F-actin reorganization that underlie internalization of TJ proteins.

MATERIALS AND METHODS

Antibodies and Other Reagents

The following primary polyclonal (pAb) and monoclonal (monoclonal antibody [mAb]) antibodies were used to detect TJ, cytoskeletal, and signaling proteins by immunofluorescence labeling and Western blotting: anti-occludin and JAM-A pAbs (Zymed Laboratories, San Francisco, CA); anti-JAM-A mAb (1H2A9, Liu *et al.*, 2000); anti-mammalian nonmuscle myosin (MNMM) IIA pAb (Covance, Berkeley, CA); anti-VASP as well as anti-mono and diphosphorylated MLC pAbs (Cell Signaling Technology, Beverly, MA); anti-MLC pAb, anti-MYPT pAb and anti-phosphorylated MYPT pAb (Santa Cruz Biotechnology, Santa Cruz, CA); anti-villin, anti-syntrophin 4, anti-ROCK pAb, and anti-ezrin mAbs (BD Pharmingen, San Diego, CA); and anti-syntrophin 3 pAb and anti-MLCK mAb (Sigma Chemical Co., St. Louis, MO). Actin-related protein 3 pAb was generously provided by Dr. Matthew Welch (University of California, Berkeley, CA). Phalloidin, as well as goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to Alexa-488, Alexa-546, or Alexa-633 dyes were obtained from Molecular Probes (Eugene, OR); horseradish peroxidase-conjugated goat anti-rabbit, goat anti-mouse, and donkey anti-goat secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Latrunculin B and W-7, Y-27632, and tannic acid were obtained from Sigma; jasplakinolide was purchased from Calbiochem (La Jolla, CA); S(-)-blebbistatin was obtained from Toronto Research Chemicals (North York, Canada). PhosphoSafe extraction buffer was purchased from EMD Biosciences (San Diego, CA). Other reagents were of the highest analytical grade and were obtained from Sigma.

Cell Culture and IFN- γ Incubation

T84 epithelial cells (ATCC, Rockville, MD) were grown in 1:1 DMEM and Hams' F-12 medium supplemented with 15 mM HEPES (pH 7.5), 14 mM NaHCO₃, antibiotics, and 6% newborn calf serum (Madara, 1987). T84 cells were seeded on collagen-coated, permeable polycarbonate filters (5- μ m pore size) with surface areas of 0.33 and 5 cm² (Costar, Cambridge, MA) as described (Madara *et al.*, 1992).

IFN- γ (100 U/ml; kind gift of Genentech, San Francisco, CA) was added basolaterally to monolayers for varying periods of time ranging from 12 to 48 h. This concentration of IFN- γ is at the bottom of a concentration curve (100–1000 U/ml), which has been reported to exert a variety of effects on intestinal epithelial cells *in vitro* (Madara and Stafford, 1989). Control monolayers were incubated with cell culture medium only.

Pharmacological Modulation of the Cytoskeleton

T84 monolayers were first incubated for indicated time with or without IFN- γ in complete medium with subsequent addition of actin or myosin-binding agents for the specified period. Stock solutions of water-insoluble chemicals were prepared in dimethyl sulfoxide (DMSO) and diluted in cell culture medium immediately before each experiment. The final concentration of DMSO was 0.1% and was included in appropriate vehicle controls.

Internalization of Plasma Membrane Proteins

Internalization of plasma membrane proteins was analyzed using surface biotinylation technique as described previously (McCormick *et al.*, 1997; Muza-Moons *et al.*, 2003). Briefly, T84 monolayers preincubated with IFN- γ for 36 h were washed in ice-cold Hanks' balanced salt solution (HBSS⁺) two times and cooled to 4°C. The apical or basolateral surface of monolayers was selectively biotinylated by application of sulfolsuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate (EZ-link sulfo-NHS-SS-Biotin; Pierce Biochemical, Rockford, IL), dissolved in HBSS⁺ at 0.5 mg/ml to the apical and basolateral compartments, respectively, for 20 min at 4°C. The reaction was quenched using 50 mM NH₄Cl in HBSS⁺ for 20 min at 4°C. Subsequently cells were washed three times with ice-cold HBSS⁺ and incubated with IFN- γ -containing medium for additional 4 h at 37°C to allow internalization of biotinylated proteins. Thereafter, cells were fixed with 3.7% paraformaldehyde (PFA), permeabilized with Triton X-100 (TX-100), and double-labeled with rhodamine-streptavidin and Alexa-488-conjugated phalloidin.

Internalization of Fluorescently Labeled Dextran

A macropinocytosis marker, lysine-fixable rhodamine-dextran (1 mg/ml), was dissolved in ice-cold medium and added to the apical side of T84 monolayers incubated with or without IFN- γ . Monolayers were kept at 4°C for 30 min to allow dextran accumulation on the cell surface and then incubated for 1 h at 37°C to induce endocytosis. Cells were fixed with 3.7% PFA, permeabilized with 0.5% TX-100, and labeled for occludin and F-actin.

Immunofluorescence Labeling

T84 monolayers incubated with or without IFN- γ were rinsed twice with ice-cold HBSS⁺. Cells were fixed/permeabilized in absolute ethanol for 20 min at -20°C followed by blocking in HBSS⁺ containing 1.5% bovine serum albumin (blocking buffer) for 60 min at room temperature and incubated for 60 min with primary antibodies in blocking buffer. Cell monolayers were then washed and incubated for 60 min with Alexa dye-conjugated secondary antibodies. Monolayers were rinsed in buffer and mounted on slides with SlowFade Antifade kit (Molecular Probes). For double labeling of myosin II with F-actin, monolayers were fixed in 3.7% PFA, permeabilized with 0.5% TX-100, and sequentially stained with primary and Alexa-546 dye-conjugated secondary antibodies. F-actin was labeled with Alexa-488-phalloidin. Stained monolayers were analyzed using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging, Thornwood, NY) coupled to a Zeiss Axio-plan 2e with 100 $\times$ Pan-Apochromat oil lens. Fluorescent dyes were imaged sequentially in frame-interlace mode to eliminate cross-talk between channels. Images shown are representative of at least three experiments, with multiple images taken per slide.

Immunoblotting

To determine expression of various cytoskeletal and regulatory proteins, T84 monolayers were scraped into a Relax buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM HEPES, pH 7.4) containing 1% TX-100, and a protease inhibitor cocktail (1:100, Sigma). To determine the amount of phosphorylated proteins, cells were homogenized in the PhosphoSafe extraction buffer. All homogenates were cleared by low-speed centrifugation (1500 $\times$ g, 5 min, 4°C) and immediately boiled in SDS sample buffer. PAGE and immunoblotting were conducted by standard protocol with 10–20 μ g of total equalized protein (measured by BCA protein quantification assay (Pierce Biochemical), per well). To ensure the equal protein loading membranes were striped and reprobed for actin. The results are shown as representative immunoblots of three independent experiments. Quantification of protein expression was performed by densitometric analysis of Western blot images using the UN-

Figure 1. IFN- γ induces internalization of occludin into F-actin-coated vacuoles in intestinal epithelial cells. T84 cells were incubated with IFN- γ for 48 h and then labeled for F-actin and either occludin or JAM-A. Reconstructed confocal images in the *x-z* and *x-y* plane revealed internalization of occludin (A; arrowheads), as well as JAM-A (B; arrowheads), into subapical F-actin-coated vacuoles (arrows). Scale bar, 10 μ m.

SCAN-IT automated digitizing software (Silk Scientific, Orem, UT). Protein expression in IFN- γ -treated samples was calculated relatively to untreated controls, which were considered as 100%.

Rho Activation Assays

To test activation status of Rho GTPases in T84 intestinal epithelial cells upon IFN- γ treatment, a rhotekin-binding assay was performed as described previously (Hopkins *et al.*, 2003). Briefly, confluent T84 monolayers were incubated with media $\pm$ IFN- γ for 38 h and harvested into Rho lysis buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 1% T-X 100, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol) protease inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktails 1 and 2 (both at 1:200, Sigma). After centrifugation to remove cellular debris, lysates from control and IFN- γ -treated cells containing equal protein concentrations (10–20 μ g according to BCA protein assay) were rotated for 45 min with 50 μ l slurry of a GST-fusion protein composed of the Rho-binding domain of the specific Rho effector rhotekin coupled to agarose beads (Upstate Biotechnology, Lake Placid, NY). Addition of GTP γ S to additional whole-cell lysates served as a positive control for Rho activation. Beads were collected by centrifugation and washed three times with lysis buffer. Then, beads were resuspended in the Relax buffer containing 20 mM of dithiothreitol, boiled for 5 min, and subjected to SDS-PAGE on a 12% Tris-HCl gel. To determine the total Rho protein expression in both control and IFN- γ -treated cells, the whole-cell lysates were run in parallel with samples eluted from Rhotekin-agarose beads. Separated proteins were transferred to nitrocellulose membrane and probed with mAb to Rho.

Statistics

Numerical values from individual experiments were pooled and expressed as mean $\pm$ SE of the mean throughout. Values obtained for control and IFN- γ -treated groups were compared by two-tailed Student's *t* tests, with statistical significance assumed at *p* < 0.05.

RESULTS

IFN- γ Induces Internalization of TJ Transmembrane Proteins into F-actin-coated Vacuoles

Because classical macropinosomes represent F-actin-coated vacuoles (Swanson and Watts, 1995), we first investigated

whether internalized TJ transmembrane proteins are associated with F-actin-rich intracellular structures in IFN- γ -treated epithelial cells. For these experiments we double-labeled T84 epithelial cell monolayers for TJ proteins occludin, JAM-A (Figure 1), and claudin-1 (unpublished data) with F-actin. In control cells, F-actin was mostly localized at the apical part of the cell representing a perijunctional F-actin ring at the level of the TJs as well as cytoskeleton of the apical brush border. In addition, a significant fraction of actin microfilaments was visualized along the lateral plasma membrane. Incubation of T84 cells for 48 h with IFN- γ caused dramatic reorganization of actin cytoskeleton. The most striking feature was appearance of large (1–5- μ m diameter) F-actin-coated vacuoles in the subapical cytosolic compartment (Figure 1; arrows). Interestingly, internalized TJ proteins, occludin, JAM-A (Figure 1; arrowheads), and claudin-1 (unpublished data) appeared to be trapped inside these F-actin-coated vacuoles.

To investigate whether the appearance of F-actin-coated vacuoles and internalization of TJ proteins are synchronized in time, we investigated the time course for both processes. In agreement with our previous study (Bruewer *et al.*, 2005), we observed the first signs of TJ protein endocytosis after 38 h of IFN- γ incubation, and this effect was accentuated after 48 h of the cytokine exposure (Bruewer *et al.*, 2005). The appearance of subapical F-actin-coated vacuoles paralleled internalization of TJ proteins (Figure 2) suggesting that these two processes are or may be casually connected.

To verify that the TJ proteins containing, F-actin-coated vacuoles represent true macropinosomes as we observed in our previous study (Bruewer *et al.*, 2005), we investigated whether these structures also incorporate a fluid phase marker, fluorescently labeled dextran. We found that in T84

Figure 2. Time course of IFN- γ -induced internalization of occludin and development of subapical F-actin-coated vacuoles. Confluent T84 monolayers were incubated with 100 U/ml IFN- γ for 12–48 h. Internalization of occludin (arrowheads) into F-actin-coated vacuoles (arrows) was determined by fluorescence labeling and confocal microscopy. After 24 h of IFN- γ incubation, organization of occludin and F-actin was analogous to that observed in polarized control epithelial cells. The first evidence of occludin internalization into F-actin-coated vacuoles was observed after 38 h of IFN- γ incubation. This effect was augmented at 48 h of IFN- γ incubation. Scale bar, 10 μ m.

cells preincubated for 38 h with IFN- γ , rhodamine-dextran accumulated in F-actin-coated vacuoles where it frequently colocalized with internalized TJ proteins (unpublished data).

Large F-actin-coated subapical vacuoles referred to as vacuolar apical compartments (VAC) have been previously reported in kidney epithelial cells that have lost cell polarity (Vega-Salas *et al.*, 1987a, 1987b; Brignoni *et al.*, 1993) and in intestinal epithelial cells of patients with a rare inherited condition termed microvilli inclusion disease (Ameen and Salas, 2000). In previous reports, VACs have been described as large vacuoles (1–6 μ m) containing microvilli and specific apical plasma membrane proteins and lacking basolateral plasma membrane proteins. In nonpolarized MDCK cells, the apical plasma membrane marker syntaxin-3 has been shown to localize in VACs, whereas a basolateral plasma membrane marker, syntaxin-4, was excluded (Low *et al.*, 2000). To investigate whether the F-actin-coated vacuoles in IFN- γ -treated T84 cells represent “classic” VACs, we immunolabeled them for different apical and basolateral proteins such as villin, ezrin, syntaxin-3 and syntaxin-4. In cells that were incubated with IFN- γ for 48 h, we observed localization of villin (Figure 3A), ezrin (unpublished data) and syntaxin-3 (Figure 3A) in the F-actin-coated vacuoles. However, these structures did not contain the basolateral marker, syntaxin-4 (Figure 3A). To further define the origin of these vacuoles, we performed experiments to analyze internalization of selectively biotinylated apical or basolateral plasma membrane proteins in T84 cells preincubated for 36 h with IFN- γ . We found that only apically associated biotin label accumulated in VACs, whereas these structures were devoid of basolaterally biotinylated proteins (Figure 3B). Finally, we confirmed the apical plasma membrane origin of VACs by using polarized application of membrane impermeant inhibitor of endocytosis, tannic acid (Polishchuk *et al.*, 2004). We first tested selectivity of tannic acid on control T84 monolayers and found that apical but not basolateral addition of this inhibitor blocked internalization of fluorescently labeled cholera toxin B from the apical plasma membrane, whereas basolateral but not apical addition of tannic acid effectively prevented endocytosis of transferrin from the basolateral

plasma membrane (our unpublished observations). In T84 cells preincubated for 36 h with IFN- γ , apical application of 0.1% (wt/vol) tannic acid for an additional 4 h completely inhibited the formation of F-actin-coated vacuoles (Figure 3C) and internalization of TJ proteins (our unpublished observation). In contrast, basolateral application of tannic acid did not affect the IFN- γ induced macropinocytosis (Figure 3C). Collectively, these data strongly suggest that IFN- γ induces internalization of TJ proteins in T84 cells by triggering selective F-actin-dependent vacuolarization of the apical plasma membrane.

Formation of VACs Is Not Mediated by a Rapid Turnover of Actin Microfilaments

Next we investigated mechanisms of F-actin reorganization that underline vacuolarization of the apical plasma membrane and endocytosis of TJ proteins. Two such mechanisms can be envisioned: one involving rapid turnover (depolymerization/repolymerization) of actin microfilaments and another involving myosin II-mediated contraction (Ivanov *et al.*, 2004a). We tested the role for rapid F-actin turnover in the formation of VACs by several experimental approaches. To block turnover of actin microfilament we used a cell permeable drug jasplakinolide, that selectively binds to F-actin and dramatically decreases the rate of actin depolymerization (Bubb *et al.*, 1994, 2000; Cramer *et al.*, 2002). T84 cells were preincubated for 37 h with IFN- γ followed by incubation with jasplakinolide (1 μ M) for an additional hour. We observed that jasplakinolide treatment failed to prevent the formation of VACs and endocytosis of TJ proteins occludin (Figure 4) and JAM-A. As a positive control for this experiment, we recently showed that the same concentration of jasplakinolide attenuated formation of contractile F-actin rings in calcium-depleted T84 cells (Ivanov *et al.*, 2004a). Next we probed if the VACs represent areas with rapid F-actin turnover using a G-actin-binding drug latrunculin B. Sequestration of monomeric actin with latrunculin B was shown to inhibit de novo actin polymerization and to rapidly disassemble actin microfilaments with high rates of turnover (Morton *et al.*, 2000). We allowed VACs to develop by incubation with IFN- γ for 40 h and then treated cells for

Figure 3. IFN- γ induced apical vacuoles are derived from the apical plasma membrane. Epithelial monolayers were incubated with 100 U/ml IFN- γ for 48 h, followed by fixation and double labeling for F-actin and either villin or syntaxin 3 and syntaxin 4. (A) In IFN- γ -treated T84 cells, reconstructed images in the x-z plane reveal colocalization of villin with apical F-actin-coated vacuoles (arrows) and accumulation of syntaxin 3 but not syntaxin 4 inside the vacuoles (arrows). (B) T84 cells were preincubated for 36 h with IFN- γ followed by selective biotinylation of either apical or basolateral plasma membranes. Cells were subsequently incubated in IFN- γ -containing medium for an additional 4 h. Note the presence of biotin label in the VACs (arrows) only if biotin was applied apically. (C) T84 cells were preincubated for 36 h with IFN- γ followed by either apical or basolateral application of 0.1% (wt/vol) tannic acid for additional 4 h. Note that selective inhibition of endocytosis from the apical plasma membrane prevents formation of F-actin-coated vacuoles (arrows), whereas inhibition of basolateral endocytosis is ineffective. Scale bar, 10 μ m.

another 15 min with either latrunculin B (5 μ M) or vehicle. Figure 5 demonstrates that latrunculin B induced rapid disruption of F-actin in the apical brush-border, but had little effect on either perijunctional actin microfilaments or on F-actin-coated vacuoles (arrows). These data suggest that VACs are not composed by rapidly turning over actin microfilaments.

In addition, we investigated if the VACs contain protein machinery that is required for actin polymerization. For that, we double-labeled IFN- γ -treated T84 cells for F-actin and either an actin-related protein (Arp) 3 or vasodilator-stimulated phosphoprotein (VASP). Arp 3 is a subunit of the Arp2/3 actin-nucleating complex, whereas VASP is critical for elongation of actin microfilaments (Suetsugu *et al.*, 2002; Welch and Mullins, 2002; Sechi and Wehland, 2004). Figure 6 shows that neither Arp 3 nor VASP were enriched in VACs. Collectively, our pharmacological and immunofluorescence labeling data strongly suggest that the dynamic reorganization (depolymerization/repolymerization) of actin microfilaments is not responsible for the IFN- γ -induced formation of VACs and internalization of TJ proteins in T84 cells.

Activity of Myosin IIA Is Required for Biogenesis of VACs and Internalization of TJ Proteins

Because dynamic F-actin turnover does not appear to be essential for IFN- γ -induced development of VACs and internalization of TJ transmembrane proteins, we next investigated if myosin-driven contraction would provide the force for these processes. In intestinal epithelial cells, contraction of apical F-actin is regulated by mammalian nonmuscle myosin (MNMM) II (Mooseker, 1985). Hence, we sought to investigate whether MNMM II plays a role in invagination of the apical plasma membrane resulting in the formation of VACs. As shown in Figure 7, incubation of IFN- γ -treated cells with a selective MNMM II inhibitor blebbistatin (50 μ M) for 4 h completely prevented appearance of F-actin-coated vacuoles and internalization of occludin. Furthermore, double labeling for F-actin and a IIA isoforms of the MNMM heavy chain revealed that the F-actin coat of VACs is enriched in this motor protein (Figure 8A, arrows).

Because activity of myosin II requires association of its heavy chains with phosphorylated regulatory myosin light chains (Tan *et al.*, 1992; Bresnick, 1999) experiments were performed to

Figure 4. Inhibition of F-actin depolymerization does not affect formation of VACs and endocytosis of TJ proteins. Confluent T84 monolayers were preincubated for 37 h with IFN- γ followed by incubation with either F-actin stabilizing drug jasplakinolide (1 μ M) or vehicle for additional 1 h. Note that F-actin stabilization failed to inhibit either formation of VACs (arrows) or internalization of occludin (arrowheads). Scale bar, 10 μ m.

determine whether IFN- γ activates myosin II in T84 cells. In particular, Western blotting and immunofluorescence analyses were performed using two antibodies that specifically recognize monophosphorylated (Ser19) and diphosphorylated (Ser19/Thr18) myosin light chain (MLC). Densitometric analysis of Western blots presented in Figure 8B did not show significant differences in amount of total MLC protein between controls and cells incubated with IFN- γ for 38 h. However we observed a significant increase in the level of monophosphorylated and diphosphorylated MLC in T84 cells exposed to IFN- γ for 38 h compared with control untreated cells ($p < 0.01$).

Additionally, double-labeling experiments revealed strong colocalization of F-actin and monophosphorylated MLC (Figure 8C) as well as diphosphorylated MLC in VACs (unpublished data). Taken together these data strongly suggest that myosin II driven contraction mediates the formation of VACs and endocytosis of TJ transmembrane proteins in IFN- γ -treated cells.

RhoA/ROCK Mediate IFN- γ -induced Formation of VACs and Internalization of TJ Proteins

Next we sought to dissect the signaling pathway that leads to hyperphosphorylation of MLC and activation of myosin II

Figure 5. Inhibition of de novo actin polymerization does not disassemble VACs in IFN- γ -treated cells. Confluent T84 monolayers were preincubated for 40 h with IFN- γ followed by 15-min incubation with either G-actin sequestering drug latrunculin B (5 μ M) or vehicle. Note that inhibition of de novo actin polymerization by sequestration of G-actin does not cause disassembly of VACs in IFN- γ -treated cells (arrows). Scale bar, 10 μ m.

Figure 6. Actin-polymerizing proteins are not enriched in VACs. T84 cells were treated with IFN- γ for 48 h, fixed, and double-labeled with F-actin and either vasodilator-stimulated phosphoprotein (VASP) or actin-related protein (Arp 3). Note that neither VASP nor Arp 3 is enriched within VACs. Scale bar, 10 μ m.

in IFN- γ -treated T84 cells. We tested the role for two protein kinases, viz., myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK) that are primarily responsible for MLC phosphorylation in stimulated cells (Adelstein, 1982; Amano *et al.*, 1996). T84 cells were preincubated for 36 h with IFN- γ followed by incubation for 4 h with either Y-27632 (20 μ M) or PIK (250 μ M), which have been shown to be permeable selective inhibitors of ROCK (Walsh *et al.*, 2001) and MLCK, respectively (Zolotarevsky *et al.*, 2002). As shown in Figure 9, inhibition of ROCK prevented IFN- γ -induced formation of VACs and internalization of occludin, whereas blockage of MLCK was ineffective. Furthermore,

pharmacological inhibition of a MLCK binding partner, calmodulin, with W-7 (10 μ M), did not affect formation of VACs and internalization of TJ transmembrane protein (our unpublished observation). Interestingly, incubation of T84 cells for 38 h with IFN- γ resulted in a more than a twofold increase in expression of ROCK-1 protein, whereas expression of MLCK was not affected (Figure 10A). These data together with our pharmacological analyses suggest that ROCK is a major activator of MLC in IFN- γ -treated T84 cells. We next investigated whether expressional up-regulation is the only mechanism that may increase activity of this protein. Because ROCK is activated by Rho GTPases, we exam-

Figure 7. Myosin II activity mediates formation of VACs and endocytosis of TJ proteins in IFN- γ -treated cells. T84 cells were preincubated with IFN- γ for 36 h followed by additional 4-h incubation with either the selective nonmuscle myosin II inhibitor blebbistatin (50 μ M) or vehicle. Localization of occludin and F-actin was determined by immunofluorescence labeling/confocal microscopy. Note that inhibition of myosin II completely blocks formation of VACs and internalization of occludin in IFN- γ -treated cells. Scale bar, 10 μ m.

Figure 8. In IFN- γ -treated cells myosin II is hyperphosphorylated and is enriched at VACs. T84 cells were incubated for 48 h with IFN- γ , fixed, and double-labeled for F-actin and either MNMM IIA (A) or phosphorylated MLC (C). Note significant accumulation of both MNMM IIA and p-MLC with F-actin in VACs (arrows). Scale bar, 10 μ m. (B) Confluent T84 monolayers were incubated with medium only or IFN- γ for 40 h and lysed, and the amount of total, mono- and diphosphorylated MLC was determined by Western blotting. Note that IFN- γ treatment causes significant increase in the level of mono- and diphosphorylated MLC. Representative Western blots and results of densitometric quantification are shown. Data are presented as mean $\pm$ SE (n = 3); *p < 0.05 compared with the nontreated control.

ined the effect of cytokine treatment on the activation status of Rho. Confluent T84 intestinal epithelial cells were incubated with IFN- γ for 38 h and Rho activity was analyzed using agarose-bead-coupled constructs containing the GTPase-binding domain of Rho effector protein rhotekin. The nonhydrolyzable GTP analog GTP γ S was used as a positive control for GTPase activation. Total levels of Rho were similar in cell lysates obtained from control and IFN- γ -treated T84 monolayers (Figure 10B). However, cytokine treatment significantly increased the amount of rhotekin-bound (active) Rho (Figure 10B). Collectively, these data suggest that IFN- γ may increase ROCK activity in T84 cells by two different mechanisms, one involving expressional up-regulation of ROCK protein and another involving activation of Rho.

Finally, we sought to investigate how activation of ROCK may result in hyperphosphorylation of MLC. It is well established that ROCK can either directly phosphorylate MLC or increase MLC phosphorylation indirectly by inhibiting its dephosphorylation by myosin phosphatase (Amano *et al.*, 1996; Kimura *et al.*, 1996; Feng *et al.*, 1999; Kawano *et al.*,

1999). ROCK has been reported to inhibit myosin phosphatase by phosphorylation of one of its components termed myosin phosphatase target subunit (MYPT; reviewed in Kaibuchi *et al.*, 1999; Ito *et al.*, 2004). To investigate whether IFN- γ treatment results in hyperphosphorylation of MYPT and thus inactivation of myosin phosphatase, we compared expression of total and phospho-MYPT in control and cytokine-treated T84 monolayers. After 38 h of IFN- γ treatment we observed no changes in protein expression of either total or phosphorylated MYPT compared with control T84 cells (Figure 10C). These data suggest that inactivation of myosin phosphatase is not involved in ROCK mediated hyperphosphorylation of MLC in IFN- γ -treated T84 cells and therefore that ROCK is likely to directly phosphorylate MLC and activate myosin II.

DISCUSSION

IFN- γ disrupts barrier function of intestinal epithelial monolayers by poorly understood mechanism that involves macropinocytosis of TJ transmembrane proteins (Bruewer *et al.*,

Figure 9. Formation of VACs and endocytosis of TJ proteins depend on activity of Rho-associated kinase but not myosin light chain kinase. T84 cells were preincubated with IFN- γ for 36 h followed by additional 4-h incubation with either selective Rho-kinase inhibitor Y-27632 (20 μ M), a specific membrane permeant myosin light chain kinase inhibitor PIK (250 μ M), or vehicle. Organization of F-actin and distribution of occludin was determined by immunofluorescence labeling and confocal microscopy. Note that inhibition of Rho-associated kinase completely blocks formation of VACs and internalization of occludin, whereas inhibition of myosin light chain kinase is ineffective. Scale bar, 10 μ m.

2005). The present study was designed to investigate if IFN- γ -induced disassembly and internalization of epithelial TJs is mediated by reorganization of actin cytoskeleton. Here we reported a novel mechanism underlying endocytosis of TJ proteins. This mechanism involves a myosin II-dependent vacuolarization of the apical plasma membrane triggered by hyperphosphorylation and activation of myosin II via the Rho-ROCK pathway.

IFN- γ Induces Internalization of TJ Transmembrane Proteins into the F-actin-coated VACs

Here we report a novel effect of IFN- γ on intracellular morphology of epithelial cells, viz., the formation of large sub-apical vacuoles (Figure 1) covered with F-actin and apically enriched actin-binding proteins such as villin and ezrin (Figure 3A). Such vacuoles contained syntaxin-3, a marker of the apical plasma membrane in MDCK and T84 epithelial cells (Low *et al.*, 2000; Ivanov *et al.*, 2004a) and selectively incorporated apically but not basolaterally biotinylated plasma membrane proteins (Figure 3, A and B). In addition formation of the vacuoles was inhibited by selectively “freezing” the apical plasma membrane with tannic acid (Figure 3). IFN- γ -induced vacuoles clearly resemble so-called VACs that were initially described in epithelial cells, which have

lost cell polarity (Vega-Salas *et al.*, 1987b; Brignoni *et al.*, 1993).

VAC formation was observed *in vitro* in human intestinal and mammary epithelial cells cultivated at low concentration of extracellular calcium and *in vivo* in breast ductal carcinomas (Vega-Salas *et al.*, 1993) and intestinal epithelium of patients with a genetic disease (Ameen and Salas, 2000). We provide the first evidence that VACs can be formed in epithelial cells under inflammatory conditions. It is surprising that VACs have not been reported in several previous studies examining effects of IFN- γ on intestinal epithelial cells. (Madara *et al.*, 1988; Colgan *et al.*, 1994; Youakim and Ahdieh, 1999). It is likely that other investigators have not observed VACs because of differences in experimental conditions and methods of analysis. In particular, we believe that use of Alexa-conjugated-phalloidin and confocal microscopy in the present study resulted in significantly improved visualization of F-actin architecture compared with immunolabeling with actin antibodies and epifluorescence microscopy used by other investigators (Colgan *et al.*, 1994; Panchuk-Voloshina *et al.*, 1999; Youakim and Ahdieh, 1999). Few previous studies have shown effects of IFN- γ on the actin cytoskeleton. Thus, in microvascular endothelial cells, this cytokine was shown to induce thickening and bundling

Figure 10. IFN- γ up-regulates expression of Rho-associated kinase, activates Rho and does not affect phosphorylation of myosin phosphatase. (A) T84 monolayers were incubated without (controls) or with IFN- γ for 38 h, and protein expression of Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK) was determined by Western blotting. To ensure equal protein loading, membranes were stripped and reprobed with anti-actin antibody. IFN- γ significantly up-regulated expression of ROCK, whereas MLCK level was unaffected. Representative Western blot and results of densitometric quantification are shown. Data are presented as mean $\pm$ SE ($n = 3$); * $p < 0.05$ compared with the nontreated control. (B) Rho activation status in control and IFN- γ -treated (38 h) T84 cells was examined by Rhotekin pull-down assay as described in *Materials and Methods*. IFN- γ significantly increased amount of active RhoA without affecting a total level of this protein. Data are presented as mean $\pm$ SE ($n = 3$); * $p < 0.05$ compared with the nontreated control. (C) T84 cells were incubated with or without IFN- γ for 38 h, and amounts of total and phosphorylated myosin phosphatase target subunit (MYPT) was determined by Western blotting. IFN- γ did not influence expression of either total or phosphorylated MYPT.

of peripheral actin microfilament (Fenyves *et al.*, 1993), whereas in thyrocytes IFN- γ reportedly reduced F-actin density (Asakawa *et al.*, 1990). Interestingly, the IFN- γ receptor has been shown to be associated with the actin cytoskeleton (Ulevitch *et al.*, 1991; Cruz *et al.*, 1999). Hence it is not surprising that F-actin is an important target for intracellular IFN- γ signaling.

We observed that VACs in IFN- γ -induced T84 cells contained selectively accumulated TJ transmembrane proteins (Figure 1) strongly suggesting involvement of VACs in disassembly and internalization of epithelial TJs. The functional role for this compartment remains to be investigated. Based on the ability of "classic" VACs to recycle back to the apical plasma membrane (Vega-Salas *et al.*, 1987b; Gilbert and Rodriguez-Boulant, 1991), we speculate that these vacuoles represent a temporary depot for junctional components and apical plasma membrane proteins. Interestingly, IFN- γ did not cause up-regulation of either clathrin-mediated internalization of transferrin or caveolar-mediated endocytosis of Cholera Toxin B in T84 cells (our unpublished observation). Hence, internalization of TJ proteins into VACs reflects specific effect of IFN- γ on T84 cells.

Myosin II Provides Force for the Formation of VACs and Endocytosis of TJ Transmembrane Proteins

F-actin-coated vacuoles in IFN- γ -treated T84 cell reminiscent of contractile F-actin rings have been observed in a variety of physiological and pathophysiological processes including cytokinesis, granule exocytosis, extrusion of apoptotic cells, and wound healing (Noguchi *et al.*, 1995; Mandato and Bement, 2001; Rosenblatt *et al.*, 2001; Sokac *et al.*, 2003). Two different mechanisms have been implicated in the formation of such contractile rings involving rapid turnover of F-actin and myosin II-dependent translocation of actin microfilaments (Ivanov *et al.*, 2004b). These mechanisms are not mutual exclusive, and the relative contribution of each probably differs, depending on experimental conditions and cell type. In our model, we did not find evidence supporting a role for F-actin turnover (depolymerization/de novo polymerization) in the formation of VACs. This compartment appeared to be quite resistant to sequestration of G-actin with latrunculin B (Figure 6) and it did not selectively accumulate actin-polymerizing machinery. Likewise, formation of VACs and internalization of TJ proteins were not affected by stabilization of intracellular F-actin with jasplakinolide (Figure 5). These data suggest that formation of VACs and endocytosis of TJ transmembrane proteins occur independently of rapid F-actin turnover.

We did however find that IFN- γ -induced formation of VACs and endocytosis of TJ proteins depends on activity of the myosin II motor as demonstrated by our observation that the myosin II inhibitor blebbistatin completely blocked formation of VACs and that activation of myosin II accumulated within the F-actin coat of VACs (Figure 8). Interestingly, the exclusive myosin II dependence of VACs formation distinguishes it from contractile F-actin rings that mediate internalization of apical junctions in calcium-depleted T84 cells (Ivanov *et al.*, 2004a). The latter structures are reportedly formed by cooperation between de novo F-actin polymerization and myosin II activity. In fact, it is likely that VAC formation may be mediated by mechanisms similar to closure of small epithelial wounds (Bement *et al.*, 1993; Florian *et al.*, 2002).

Role of Rho/ROCK in IFN- γ -induced Formation of VACs and Internalization of TJ Proteins

Phosphorylation of MLC and activation of myosin II can be mediated by several intracellular kinases, among which ROCK and MLCK are thought to be especially important (Adelstein, 1982; Amano *et al.*, 1996). Our pharmacological analysis strongly suggests that ROCK but not MLCK is involved in MLC phosphorylation that triggers formation of VACs and endocytosis of TJ transmembrane proteins in IFN- γ -treated T84 cells (Figure 9). In agreement with these data, we observed that IFN- γ up-regulated expression of ROCK protein and stimulated activity of its upstream regulator, Rho (Figure 10). ROCK was shown to either directly phosphorylate MLC at Ser19 and to a lesser extent, Thr18 (Amano *et al.*, 1996) or increase level of MLC phosphorylation indirectly, by phosphorylating MYPT and thus inhibiting myosin phosphatase (Hartshorne, 1998; Feng *et al.*, 1999; Kawano *et al.*, 1999). In the present study, we observed that increased level of mono- and diphosphorylated MLC was not accompanied by hyperphosphorylation of MYPT in IFN- γ -treated cells (Figure 10). We concluded therefore that direct phosphorylation of MLC by ROCK represents the major mechanism underlying activation of myosin II in IFN- γ -treated T84 cells. This mechanism appears to be different from those suggested to explain barrier disruption in Caco-2 epithelial cells either exposed to enteropathogenic *Escherichia coli* or treated by a combination of IFN- γ and TNF- α (Zolotarevsky *et al.*, 2002). In Caco-2 monolayers, pathogen induced phosphorylation of MLC was blocked by a cell-permeable oligopeptide, PIK, which specifically inhibits MLCK. In contrast, this inhibitor (Figure 9) as well as calmodulin blocker, W-7 (our unpublished observation) had no effect on formation of VACs and internalization of TJ proteins in IFN- γ -treated T84 cells. These data suggest that different cells may employ different intracellular cascades in response to similar extracellular stimuli.

In conclusion, the present study demonstrates that IFN- γ induces endocytosis of TJ transmembrane proteins by triggering selective vacuolarization of the apical plasma membrane. Formation of VACs in IFN- γ -treated cells is mediated by myosin II-driven contraction associated with stimulation of Rho activity and up-regulation of ROCK.

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