Peroxisome proliferator-activated Receptor γ (PPARγ) causes epithelial to mesenchymal transformation (EMT) in intestinal epithelial cells, as evidenced by reorganization of the actin cytoskeleton, acquisition of a polarized, mesenchymal cellular morphology, increased cellular motility, and colony scattering. This response is due to activation of Cdc42, resulting in p21-activated kinase-dependent phosphorylation and activation of MEK1 Ser298 and activation of ERK1/2. Dominant negative MEK1, MEK2, and ERK2 block PPARγ-induced EMT, whereas constitutively active MEK1 and MEK2 induce a mesenchymal phenotype similar to that evoked by PPARγ. PPARγ also stimulates ERK1/2 phosphorylation in the intestinal epithelium in vivo. PPARγ induces the p110α subunit of phosphoinositide 3-kinase (PI3K), and inhibition of PI3K blocks PPARγ-dependent phosphorylation of MEK1 Ser298, activation of ERK1/2, and EMT. We conclude that PPARγ regulates the motility of intestinal epithelial cells through a mitogen-activated protein kinase cascade that involves PI3K, Cdc42, p21-activated kinase, MEK1, and ERK1/2. Regulation of cellular motility through Rho family GTPases has not been previously reported for nuclear receptors, and elucidation of the mechanism that accounts for the role of PPARγ in regulating motility of intestinal epithelial cells provides fundamental new insight into the function of this receptor during renewal of the intestinal epithelium.

The nuclear receptor peroxisome proliferator-activated receptor (PPAR)γ1 is expressed at high levels in many cells of epithelial origin, including those of the gastrointestinal tract (1, 2). PPARγ is activated by the thiazolidinedione class of drugs (3), which inhibit azoxymethane-induced colon carcinogenesis in wild type mice (4, 5). Furthermore, hemizygous knock-out of PPARγ exacerbates colon tumor formation in azoxymethane-treated mice (6). Paradoxically, thiazolidinediones appear to promote intestinal tumor growth in mice that harbor mutations in the adenomatous polyposis coli (APC<sup>wt/min</sup>) gene (7, 8), and long term treatment with high concentrations of thiazolidinediones induces caecal tumors in mice (9, 10). These observations suggest that PPARγ may, under different circumstances, function as a tumor suppressor or as a tumor promoter. However, little is known about the physiological role of PPARγ in the gastrointestinal epithelium, which makes it difficult to construct testable hypotheses concerning those aspects of PPARγ signaling that may account for these paradoxical effects of thiazolidinediones.

To address these questions, we carried out a series of studies to elucidate the functions of PPARγ in nontransformed gastrointestinal epithelial cells. Our initial studies indicate that PPARγ plays a critical role in a number of processes that are central to renewal of the intestinal epithelium (11). The intestinal epithelium is one of the most dynamic tissues in the adult. The entire epithelium is replaced every 3 days or so by a highly orchestrated process that involves proliferation, migration, and exfoliation. Proliferation is restricted to a population of transit amplifying cells located within the crypts of Lieberkühn. These cells are extruded from the crypts, withdraw from the cell cycle, and differentiate into mature villus epithelial cells. The transition from transit amplifying to villus epithelial cells occurs at the crypt/villus junction. Mature villus epithelial cells then migrate to the villus tips where they dissociate from the epithelium and the underlying basement membrane and are shed into the intestinal lumen. Thus, transit amplifying cells are characterized by their proliferative capacity, whereas mature villus epithelial cells are characterized by a high degree of motility and ultimately by decreased cellular adhesion. PPARγ is expressed at low levels in the transit amplifying cells but is induced at the crypt/villus junction, suggesting that PPARγ might play a role in epithelial migration and/or adhesion.

To test this hypothesis, we analyzed the effects of PPARγ in nontransformed rat intestinal epithelial (RIE) cell lines in culture. RIE cells are derived from proliferative crypt cells of embryonic rat bowel (12), and, like the transit amplifying cells from which they are derived, RIE cells express low levels of PPARγ. We engineered these cells to express PPARγ to recapitulate the transition that occurs at the crypt/villus junction and thereby test the hypothesis that induction of PPARγ is...
linked to differentiated functions of mature villus epithelial cells. A genomic analysis revealed that PPARγ regulates a large cohort of genes that are involved in establishment and maintenance of the cytoskeleton and in cellular motility (11). Activation of PPARγ results in acquisition of a polarized mesenchymal morphology, which is associated with increased directional motility, colony scattering, and decreased cellular adhesion (11). These observations indicate that PPARγ induces epithelial to mesenchymal transformation (EMT) in intestinal epithelial cells. EMT is a complex process that involves acquisition of a polarized mesenchymal morphology, associated with alteration in the structure of the cytoskeleton caused by activation of Rho family GTPases. EMT is associated with increased cellular motility and decreased cellular adhesion, and it is thought that EMT reflects in vitro those processes that are involved in epithelial motility in vivo. Induction of EMT is a well known response to polypeptide factors such as hepatocyte growth factor and transforming growth factor β (TGF-β). However, induction of EMT by a nuclear receptor has not previously been reported, and the mechanism that accounts for such a response is unknown.

Here we have elucidated the mechanism that accounts for the morphogenetic effects of PPARγ in intestinal epithelial cells. PPARγ activates the Rho family GTPase Cdc42 by a phosphoinositide 3-kinase (PI3K)-dependent mechanism. Furthermore, our data indicate that the PPARγ-mediated morphogenetic response involves activation of ERK1/2 downstream of PK1 and MEK1. These studies represent the first demonstration of a mechanistic link between activation of a nuclear receptor and regulation of cellular motility through PI3K, Rho family GTPases, and MAPKs. Elucidation of this mechanism provides new insight into the physiological role of PPARγ in renewal of the intestinal epithelium. Furthermore, the ability of PPARγ to induce EMT suggests one possible mechanism that may account for the ability of this receptor to promote tumorigenesis under certain circumstances.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagent**—U0126, LY294202, and Me2SO were purchased from Sigma-Aldrich. U0124, PD98059, and Y27632 were purchased from Calbiochem. Hygromycin B and Geneticin (G418) were from Invitrogen. TGF-β1 was from R & D System. Vehicle for TGF-β1 (4 mM HCl, 0.1% bovine serum albumin) was prepared according to the manufacturer’s instructions. Troglitazone was obtained from Sigma-Aldrich. Rosiglitazone and 15-D-12,14-prostaglandin J2 were obtained from Cayman, and pioglitazone was a gift from Dr. John A. Copland (Mayo Clinic). RS5444 was obtained from Sankyo Co., Ltd. This thiazolidinedione was originally developed for treatment of diabetes (13), activates PPARγ but not PPARα or PPARδ (11), and has anti-tumor activity (14). Unless otherwise stated, the cultures were treated with 10 mM RS5444 in Me2SO, and control cultures received 0.1% Me2SO.

**Cell Culture and Plasmids**—RIE/S3 and RIE/S3γ1 cells were grown in 5% charcoal dextran-treated fetal bovine serum in Dulbecco’s modified Eagle’s medium, as previously described (11). The cells were placed in serum-free Dulbecco’s modified Eagle’s medium for 16 h before the addition of RS5444. Full-length mouse PPARγ cDNA was subcloned into the retroviral expression vector pLNCX. Mutation at C1295 was introduced using a QuikChange II XL site-directed mutagenesis kit (Stratagene). Stably transformed populations expressing wild type and C1295 mutant PPARγ were isolated by drug selection. Hemagglutinin-tagged wild type (WT), constitutively active, and kinase dead rat MEK1 or MEK2 constructs, FLAG-tagged WT and dominant negative (DN) mouse ERK2 constructs, and Myc-tagged dominant negative mouse PAK1 were obtained from Dr. Scott Eblen (University of Virginia). These were subcloned into the pLNCX retroviral expression vector. Retroviruses encoding Myc-tagged dominant negative RhoA and Rac1 were provided by Dr. Panos Anastasiadis. DN Rac1, DN RhoA, and DN PAK1 were cloned in the pLZRS retroviral expression vector, which encodes green fluorescent protein. RIE/S3γ1 cells were transiently infected with these retroviruses, and the efficiency of infection in all cases was >90%, as assessed by green fluorescent protein expression.

**Protein Preparation and Western Blotting Analysis**—The cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed by sonication in protein lysis buffer consisting of 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 100 mM NaF, and a protease inhibitor mixture (Sigma-Aldrich). Protein concentration was determined by the method of Bradford using the Bio-Rad protein assay reagent. An aliquot of total protein (5 µg) was resolved by electrophoresis in 10% Tris-glycine gels (Invitrogen) and trans-
ferred to polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 5% nonfat milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20 (TBST) overnight at 4 °C. Thereafter, the membrane was incubated with antibodies to PPARγ (Santa Cruz), phospho-MEK1 Ser298 (Cell Signaling), phospho-ERK1/2 Thr202/Tyr204 (Cell Signaling), ERK1/2 (Cell Signaling), phospho-RSK1 Thr359/Ser363 (Cell Signaling), RSK1 (Cell Signaling), phospho-FAK Ser 910 (BIOSOURCE), FAK, hemagglutinin (BD), FLAG (Sigma-Aldrich), p110α (Cell Signaling), p85 (Cell signaling), PTEN (Cell Signaling), phospho-Akt T423 (Cell Signaling), Akt (Cell signaling), or actin (Santa Cruz) diluted in 1% milk/TBST at room temperature for 2 h, after which the membrane was washed in TBST three times for 10 min each. The membrane was then incubated with horseradish peroxidase-conjugated anti-mouse (Santa Cruz), anti rabbit (Cell signaling), or anti-goat (Santa Cruz) secondary antibodies in 1% milk/TBST at room temperature for 1 h. The membrane was washed three times in TBST for 10 min each. Antigen-antibody complexes were detected using the ECL Plus chemiluminescent system (Amersham Biosciences).

Rho GTPase Pulldown Assays—The activity of Rho family GTPases was measured as previously described (15). Briefly, the cells were washed once with ice-cold PBS and lysed by incubating for 5 min on ice in cold lysis buffer consisting of 20 mM HEPES, pH 7.5, 0.5% Nonidet P-40, 100 mM NaCl, 0.2% deoxycholic acid, 10% glycerol, 10 mM MgCl2, and protease inhibitor mixture. The extracts were centrifuged at 13,000 rpm for 5 min at 4 °C. An aliquot of the supernatant (20 μl) was saved for protein quantitation (as described above) and loading as whole cell lysate. The rhotekin Rho-binding domain bound to glutathione-agarose beads (Upstate Biotechnologies, Inc.) was used to isolate Rho-GTP, according to the manufacturer’s instruction. The p21-binding domain of human PAK-1 bound to glutathione-agarose beads (Upstate Biotechnologies, Inc.) was used to isolate Rac1-GTP and Cdc42-GTP. The amount of RhoA-, Rac1-, or Cdc42-GTP bound to the corresponding beads, and the amounts of total RhoA, Rac1, and

FIGURE 2. Activation of PPARγ induces scattering of RIE cells. A, RIE/S3 (panels 1 and 2) and RIE/S3 γ1 (panels 3 and 4) cells were incubated with 0.1% Me2SO (panels 1 and 3) or 10 mM RS5444 (panels 2 and 4) for 24 h. Treated cells were fixed and stained. The bar equals 30 μm. B, RIE/S3 cells were cultured for 24 h in conditioned medium from RIE/S3 γ1 cells that had been grown for 24 h in RS5444. The bar equals 20 μm. C, RIE cells were transfected with either WT PPARγ or PPARγ C129C. PPRE3-TK-Luc reporter activities were measured following a 24-h treatment of Me2SO or RS5444. The data represent the normalized means ± S.D. (n = 3). Western blotting was used to measure PPARγ and actin expression in RIE cells stably expressing WT PPARγ or PPARγ C129S. D, RIE cells expressing either wild type or C129S PPARγ, as shown in C, were treated for 24 h with RS5444 and stained. The bar equals 20 μm. DMSO, dimethyl sulfoxide.
Cdc42 were quantitated by Western blotting with anti-RhoA (Santa Cruz), anti-Rac1 (BD), or anti-Cdc42 (BD) antibodies as described above.

Transient Transfection Assay—pLNCX/PPARγ, pLNCX/PPARγC129S, and/or PPRE3-TK-Luc were cotransfected with phRL-SV40 (Promega) into subconfluent RIE cells in 6-well plates using FuGENE 6 (Roche Applied Science) at a DNA:liposome ratio of 1:3. Transfection was carried out in serum-free Dulbecco’s modified Eagle’s medium overnight. Fresh medium was added along with appropriate treatment. The cells were harvested after 24 h. Total cell extracts were prepared for dual luciferase assay according to the manufacturer’s instructions (Promega) using a Veritas microplate luminometer (Turner Biosystems). The activity of Renilla luciferase was used as an internal control. The results are expressed as the means of triplicate determinations ± standard deviations.

Immunocytochemistry—The cells grown on cover glasses were fixed in 3% paraformaldehyde for 30 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with Antibody Diluent (DAKO) for 30 min. The cover glasses were then incubated with a nonspecific rabbit polyclonal antibody (Biomol) diluted in Antibody Diluent for 1 h, after which the cover glasses were washed three times with PBS/0.05% Tween 20 (PBST). The cover glasses were then incubated with Biotinylated Link Universal and streptavidin-horseradish peroxidase from the DakoCytomation LSAB+ system-horseradish peroxidase kit (DAKO), according to the manufacturer’s instruction. Following three times of PBST wash, the cells were stained with a DAKO liquid DAB+ substrate-chromogen system (DAKO). Cover glasses were mounted on glass slides using Poly.Mount Xylene (Polysciences). The cells were observed with an Olympus BX51/52 system microscope using a 20x objective. The images were captured using Olympus DP70 microscope digital camera and digital image capture software and compiled into Adobe Photoshop. Alternatively, the cells cultured on cover glasses were fixed in 3% paraformaldehyde for 30 min, blocked with 3% nonfat milk/PBS for 10 min, and incubated with Texas Red-X phalloidin (Molecular Probes) for 30 min in the dark. The cover glasses were then washed three times with PBS and mounted on glass slides using Aqua Poly/Mount (Polysciences). The cells were visualized under a Leica DM5000B fluorescent microscope using a 60x planApo Leica objective.

FIGURE 3. PPARγ induces a polarized morphology but does not promote actin stress fiber formation. A, RIE/S3γ1 cells (panels 1–4) were treated for 24 h with either 10 nM RS5444 or 1 ng/ml TGF-β1. IEC/γ1 cells (panels 5 and 6) were treated with 100 nM rosiglitazone for 24 h. The cells were fixed and stained with Texas Red-X phalloidin to visualize the actin cytoskeleton. B, RIE/S3γ1 cells were treated 24 h with Me2SO (panel 1), 10 nM RS5444 (panel 2), 6 μM troglitazone (panel 3), 100 nM rosiglitazone (panel 4), 1 μM 15-Δ12,14-prostaglandin J2 (panel 5), or 200 nM piagliatzone (panel 6). The cells were stained with Texas Red-X phallolidin. The bars equal 10 μm. DMSO, dimethyl sulfoxide.
Photos were acquired with the FX4000 program using a DFC350FX CCD camera and compiled in Photoshop.

Male adult mice (6–8 weeks) were used for PPARγ immunohistochemistry. For analysis of ERK1/2 phosphorylation, two age-matched female mice received 10 mg/kg/day RS5444 by oral gavage for 3 days, and two mice received diluent (carboxymethyl cellulose). All of the mice were anesthetized by CO2, followed by cervical dislocation. Then 4% paraformaldehyde in ice-cold PBS was perfused through the left ventricle. The whole small bowel from duodenum to ileum was isolated. Luminal contents were flushed briefly with PBS and suspended in 4% paraformaldehyde in PBS for 4 h. The tissues were then dehydrated, embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization and rehydration, the sections were fully immersed in DAKO target retrieval solution and steamed for 20 min. The sections were cooled for 30 min at room temperature before further processing. After quenching with 1% hydrogen peroxide in methanol, the sections were blocked with DAKO antibody diluent, and incubated with antibody against PPARγ (Cell Signaling) or phospho-ERK1/2 (Cell Signaling) overnight at 4 °C. The sections incubated with diluent alone were used as controls. After washing with PBS, the PPARγ antibody was detected by Vectasain ABC (Vector Laboratories) and the phospho-ERK1/2 antibody by Envision Dual Link (DAKO). The sections were counterstained with hematoxylin for 10 s. Dehydrated sections were mounted on glass slides by DAKO permanent mounting medium (DAKO). All of the sections were observed under a transmission light microscope (Olympus BX51/52). The images were captured using an Olympus DP70 microscope digital camera and digital image capture software and compiled into Adobe Photoshop. Phospho-ERK1/2 nuclear staining intensity was measured using ImagePro Plus Ver5.0. Staining intensity was measured in 30 crypts and 30 villi from each of two control mice and in 42 crypts and 42 villi from each of the RS5444-treated mice. Statistical analysis of the data was carried out using the t test function of SigmaStat 32.

Live Cell Imaging—The cells were plated in glass-bottomed culture dishes (35-mm Petri dish, 14-mm Microwell, No. 0 cover glass; MatTek Corporation). A Leica AS-MDW live cell imaging work station was used to capture live cell images at 400×, every 30 min for 24 h. The photos were compiled into AVI files at three frames/s.

RESULTS

PPARγ Induces EMT of Intestinal Epithelial Cells—Renewal of the epithelium depends upon proliferation of “transit amplifying” cells within the crypts of Lieberkühn. When these cells reach the crypt/villus interface, they cease to proliferate and differentiate into absorptive epithelial cells, which migrate to the villus tip, where they are shed into the lumen. PPARγ is expressed at very low levels in proliferative transit amplifying crypt cells (Fig. 1A). Fetal RIE cells are proliferative enterocytes, and, like the transit amplifying crypt cells from which they are derived, RIE/S3 cells expressed very low levels of PPARγ (Fig. 1B). PPARγ expression increases dramatically as cells reach the crypt/villus junction and begin their migration to the villus tips (Fig. 1A). This observation suggests that PPARγ may play a critical role in regulating the differentiated functions of intestinal epithelial cells. To test this hypothesis, we engineered RIE/S3 cells to express mouse PPARγ1 (RIE/S3-γ1 cells; Fig. 1B), thereby recapitulating the transition that occurs at the crypt/villus interface, where transit-amplifying cells differentiate into villus epithelial cells. These RIE/S3-γ1 cells exhibited a characteristic morphological response when PPARγ was activated.

RIE/S3 cells grow as distinct colonies when seeded at low densities, indicating that such cells exhibit very little inherent motility (Fig. 2A, panel 1). RIE/S3 cells express little or no PPARγ, and no perceptible change in cellular morphology occurred when the PPARγ agonist RS5444 was added to RIE/S3 cells (Fig. 2A, panel 2). On the other hand, RIE/S3-γ1 cells, upon activation of PPARγ, acquired a characteristic polarized mes-
PPARγ Promotes EMT

FIGURE 5. RS5444 induces RhoA and Cdc42 activities. In A, RIE/S3 y1 cells were incubated with Me2SO or RS5444 for 24 h. Activities of RhoA, Rac1, and Cdc42 were measured by pulldown assays as described under "Experimental Procedures." In B, RIE/S3 y1 cells were incubated with Me2SO (panel 1), RS5444 (panels 3 and 4), or 1 μM Y27632 (panel 2) for 24 h. The cells were also treated with vehicle (panels 5 and 6). 1 ng/ml TGF-β1 (panels 7 and 8), and Y27632 (panel 6 and 8) for 24 h and then stained with Texas Red-X phalloidin. Transient expression of green fluorescent protein was indicated in parallel, and stably transduced populations were selected. As shown in Fig. 2C, cells that express PPARγ C1295 did not activate a transiently transfected PPRE/luc reporter, and such cells did not undergo colony scattering or morphological transformation upon activation of PPARγ (Fig. 2D, panels 3 and 4). This observation indicates that DNA binding is required for PPARγ-mediated cytodifferentiation of intestinal epithelial cells.

Scattering of RIE/S3 y1 colonies was accompanied by marked changes in cell shape and cytoskeletal organization. Untreated RIE/S3 y1 cells exhibited a relatively typical epithelial morphology: rounded, flattened, nonpolarized, with concentric actin rings about the nucleus (Fig. 3A, panel 1). Activation of PPARγ resulted in acquisition of a polarized, mesenchymal morphology with elaboration of distinct filopodia and lamellapodia (Panel 2). The structure of the actin cytoskeleton was markedly altered by activation of PPARγ, although we did not observe formation of pronounced actin stress fibers, as seen in TGF-β1-treated RIE/S3 y1 cells (panel 4).

To confirm that the PPARγ-mediated morphogenetic response is not restricted to RIE cells, we engineered a second rat embryonic crypt cell line, IEC-6, to express mouse PPARγ. Like RIE, IEC-6 cells express little or no PPARγ, whereas the IEC/γ derivatives express PPARγ at levels comparable with those observed in RIE/S3 y1 (data not shown). Activation of PPARγ in IEC/γ cells using rosiglitazone resulted in acquisition of a mesenchymal morphology and colony scattering, as shown in Fig. 3A (panels 5 and 6). Furthermore, cytodifferentiation of RIE/S3 y1 cells was observed when PPARγ was activated by the thiazolidinediones RS444, troglitazone, rosiglitazone, and pioglitazone, as well as the naturally occurring PPARγ agonist 15-D-D12,14-prostaglandin J2 (Fig. 3B). These data indicate that colony scattering and the associated morphological transformation that we have observed are general features of PPARγ activation in intestinal epithelial cells.

The scattering response and morphological transition that occur upon activation of PPARγ indicate that this receptor stimulates cellular motility. We have previously shown that activation of PPARγ in RIE/S3 y1 cells increases the chemotactic response to fetal bovine serum (11). We used micro-cinematography to determine whether PPARγ promoted nondirectional migration (i.e. scattering) of RIE/S3 y1 cells. The cells were

enchymal morphology and moved away from each other in a scattering response (Fig. 2A, panels 3 and 4).

Colony scattering may be caused by a number of secreted polypeptide factors, such as hepatocyte growth factor and TGF-β. We considered the possibility that PPARγ might induce expression and secretion of soluble scatter factors, resulting in an autocrine/paracrine scattering response. However, conditioned medium from RS5444-treated RIE/S3 y1 had no effect on the morphology of RIE/S3 cells (Fig. 2B, panel 2), suggesting that PPARγ-mediated cytodifferentiation is not exclusively due to elaboration of soluble scatter factors.

The observation that RIE/S3 cells do not respond to thiazolidinediones (Fig. 2A) indicates that the morphological response is absolutely dependent upon expression of PPARγ. To determine whether DNA binding by PPARγ was required for this response, we generated a mutated form of mouse PPARγ1, in which the critical forth cysteine in the first zinc finger was mutated to serine (C1295). The corresponding mutation in PPARγ2 (C1595) forms heterodimers with RXRα but cannot bind peroxisome proliferator response elements or activate transcription (16). PPARγ C1295 was stably introduced into RIE cells using retrovirus-mediated transduction. Wild type PPARγ was introduced in parallel, and stably transformed populations were selected. As shown in Fig. 2C, cells that express PPARγ C1295 did not activate a transiently transfected PPRE/luc reporter, and such cells did not undergo colony scattering or morphological transformation upon activation of PPARγ (Fig. 2D, panels 3 and 4). This observation indicates that DNA binding is required for PPARγ-mediated cytodifferentiation of intestinal epithelial cells.
treated with agonist for 24 h. Thereafter photographs were taken every half hour for 24 h, and the positions of individual cells in the field were determined and plotted in an \(x\), \(y\), \(z\) plot in which the coordinates of the cell on the stage were plotted in the \(x\) and \(y\) dimensions as a function of time in the \(z\) dimension. Representative data for untreated RIE/S3\(y1\) cells are shown in Fig. 4A. All three of these cells divided during the course of the observation, but neither the parental cells nor their progeny moved very far from their original positions, indicated in Fig. 4A by the vertical dotted lines. These data are consistent with the colony morphology of RIE/S3\(y1\) cells (Fig. 2A), which indicates that such cells exhibit little or no inherent motility. However, activation of PPAR\(\gamma\) resulted in a high degree of random movement (Fig. 4B). These observations define the cellular response of intestinal epithelial cells to activation of PPAR\(\gamma\): reorganization of the actin cytoskeleton, acquisition of a polarized mesenchymal morphology, and increased motility, leading to colony scattering. Note also that only one of the cells shown in the RS5444-treated culture divided (Fig. 4B), consistent with our previous observation that PPAR\(\gamma\) inhibits proliferation of RIE/S3\(y1\) cells (11). We conclude that PPAR\(\gamma\) inhibits proliferation and induces EMT in intestinal epithelial cells in culture.

**PPAR\(\gamma\) Promotes EMT**

**FIGURE 6.** RS5444 induces activation of MAPK cascade. A, RIE/S3\(y1\) cells were stably transfected with an “empty” retrovirus (pBabe) or a retrovirus that encodes Myc-tagged dnPAK1. Parental RIE/S3\(y1\) or dnPAK1-transfected cells were treated 24 h with 10 \(\mu\)M RS5444, extracts were prepared, and Western blotting was carried out to measure phospho-MEK1 (Ser298) and ERK1/2. In B, RIE/S3\(y1\) or RIE/S3 cells were incubated 24 h with Me\(2S\)O or RS5444 in the presence of absence of U0126 or U0124 (10 \(\mu\)M), as indicated. The cell lysates were analyzed by Western blotting using antibodies to phospho-ERK1/2 (T202/Y204), and total ERK1/2.

**PPAR\(\gamma\) Activates Rho Family GTPases**—EMT is associated with activation of Rho GTPase family members, including Rac, Rho, Cdc42, and their relatives. As shown in Fig. 5A, activation of PPAR\(\gamma\) caused an increase in RhoA activity, as evidenced by an increase in the rhotekin-precipitable, GTP-bound form of RhoA. Rho activates the downstream kinase ROCK, which is required for Rho-dependent morphological effects, including stress fiber formation (17, 18). However, the ROCK inhibitor Y27632 had no effect on PPAR\(\gamma\)-mediated colony scattering (Fig. 5B, panels 3 and 4). Y27632 inhibited stress fiber formation and caused cell spreading in RIE/S3\(y1\) cells (Fig. 5B, panels 5 and 6), and the inhibitor prevented formation of actin stress fibers in TGF-\(\beta1\)-treated RIE/S3\(y1\) cells (Fig. 5B, panels 7 and 8). Although Y27632 affects cellular morphology in the absence
of PPARγ agonist, this effect does not block PPARγ-dependent colony scattering, suggesting that Rho is not required for PPARγ-mediated cytodifferentiation. This hypothesis was confirmed in RIE/S3 γ1 cells that were engineered to transiently express a dominant negative mutant of RhoA (RhoA T19N). This mutant had no effect on the PPARγ-induced morphological change (Fig. 5C, panel 5), although DN RhoA blocked TGF-β1-induced actin stress fiber formation (panel 6). We conclude that activation of RhoA by PPARγ is not required for colony scattering.

A dominant negative mutant of Rac1 (Rac1 T17N) blocked the PPARγ-induced morphological response (Fig. 5C, panel 6) but had no effect on TGF-β1-mediated actin stress fiber formation (panel 9). These data suggest that PPARγ induces the scattering response through activation of Rac1 or Cdc42. Rac1 and Cdc42 are regulated by a common set of modifiers, including the guanine nucleotide exchange factors Tiam and Vav (reviewed in Refs. 19 and 20). Because Rac1 T17N binds to these guanine nucleotide exchange factors and blocks activation of both Rac1 and Cdc42, the data shown in Fig. 5C (panel 6) do not discriminate between a potential role of Rac and Cdc42 in PPARγ-mediated colony scattering. However, PPARγ causes an increase in the active, GTP-bound form of Cdc42 (Fig. 5A), with little or no effect on Rac1 activity. Based upon these observations, we conclude that PPARγ-induced EMT requires Cdc42 activation.

**PPARγ Promotes EMT**

**PPARγ Activates ERK1/2**—The major downstream effector of Cdc42 is PAK. PAK, upon activation by Cdc42 (and/or Rac1) phosphorylates Ser298 of MEK1 and primes MEK1 for phosphorylation by Raf-1 (21, 22). Activation of PPARγ increased Ser298 phosphorylation of MEK1 (Fig. 6A), suggesting that PPARγ activates a Cdc42 → PAK → MEK1 signaling pathway. To test this hypothesis, we transfected RIE/S3 γ1 cells with a catalytically inactive, dominant negative mutant of PAK1 (K299R). Expression of this mutant was confirmed by Western blotting to detect Myc-tagged DN PAK1 and total PAK1 (Fig. 6A). DN PAK1 inhibited PPARγ-mediated phosphorylation of Ser298 of MEK1 (Fig. 6A). Because phosphorylation of Ser298 promotes Raf-1-mediated activation of MEK1 (21, 22), we conclude that activation of PPARγ is likely to result in a PAK-mediated increase in MEK1 activity.

The only known substrate of MEK1 is ERK1/2, and PPARγ stimulates phosphorylation of ERK1 and ERK2 (Fig. 6, A and B). Phosphorylation of ERK1/2 was blocked by dnPAK1 (Fig. 6A) and by the MEK1 inhibitor U0126, but not by the inactive analog U0124 (Fig. 6B). Activation of ERK1/2 by PPARγ agonist requires PPARγ expression, as evidenced by failure to observe ERK1/2 phosphorylation in RIE/S3 cells, which lack PPARγ (Fig. 6B). ERK1/2 phosphorylation was first observed 6–12 h after activation of PPARγ (Fig. 6C). No increase in ERK1/2 phosphorylation was observed at 10 min, 20 min, or 2 h after activation of PPARγ (data not shown). The morphological response to PPARγ activation was completely blocked by the MEK1 inhibitors PD098059 and U0126 (Fig. 6D), indicating that PPARγ-mediated colony scattering is due to activation of a MEK1 → ERK1/2 pathway.

**PPARγ Stimulates ERK1/2 Phosphorylation in Vivo**—We used a phosho-ERK1/2-specific antibody to determine whether PPARγ also regulates MAPK activity in the small intestine in vivo. Phospho-ERK1/2 staining was significantly increased in the villus epithelium of agonist-treated mice, compared with controls, with no apparent change in ERK1/2 staining of stromal cells (Fig. 7A, panels 1 and 2). The increase in phospho-ERK1/2 staining was primarily restricted to the nucleus of epithelial cells (Fig. 7A, panels 3 and 4), consistent with the known properties of activated ERK1/2 (23). Quantitative analysis of the intensity of nuclear staining of p-ERK1/2 in control and RS5444-treated mice revealed a statistically significant effect in villus epithelial...
cells, but no effect on ERK1/2 phosphorylation in transit amplifying cells within the crypts (Fig. 7B). These data indicate that PPARγ activation results in increase ERK1/2 activity in intestinal villus epithelial cells in vivo, as well as RIE cells in culture.

**Activation of ERK1/2 Is Required for the PPARγ-mediated Morphological Response**—The observation that MEK inhibitors blocked PPARγ-mediated colony scattering (Fig. 6C) suggests that PPARγ regulates ERK1/2 activity through a MEK-dependent mechanism and that activation of ERK1/2 is central to the PPARγ-induced mesenchymal morphology. To confirm this hypothesis, we stably transfected RIE/S3γ1 cells with wild type, constitutively active, or kinase inactive mutants of MEK1 and MEK2. The “empty” retrovirus control (LNCX) and WT MEK1 and MEK2 had no effect on PPARγ-mediated phosphorylation of ERK1/2 (Fig. 8A). Likewise, WT MEK1 and WT MEK2 had no effect on PPARγ-mediated colony scattering (Fig. 8B). The constitutively active mutants of both MEK1 and MEK2 increased basal phosphorylation of ERK1/2, as expected (Fig. 8A), with little or no increase in ERK1/2 phosphorylation after activation of PPARγ (Fig. 8A). RIE/S3γ1 cells that stably express constitutively active MEK1/2 exhibited a polarized, mesenchymal morphology similar to that observed upon activation of PPARγ (Fig. 8B), indicating that activation of ERK1/2 signaling in these cells is sufficient to induce the mesenchymal phenotype.

Kinase inactive mutants of MEK1 and MEK2 blocked PPARγ-mediated phosphorylation of ERK1/2 (Fig. 8A). Kinase dead MEK1 and kinase dead MEK2 also completely blocked PPARγ-mediated colony scattering (Fig. 8B), indicating that ERK1/2 activation is necessary for the morphological effects of PPARγ. To test this hypothesis, we engineered RIE/S3γ1 cells to express either wild type or dominant negative (kinase inactive) FLAG-tagged ERK2. ERK1/2 activity in such cells was monitored by measuring phosphorylation of RSK1, a known ERK1/2 substrate. PPARγ stimulated phosphorylation of RSK1, consistent with increased activity of ERK1/2 (Fig. 9A). Stable expression of wild type ERK2 had no effect on basal or PPARγ-induced RSK1 phosphorylation (Fig. 9A). Furthermore, WT ERK2 had no affect on the colony scattering response (Fig. 9B). DN ERK2, on the other hand, inhibited RSK1 phosphorylation (Fig. 9A) and completely blocked PPARγ-mediated colony scattering (Fig. 9B). On the basis of these observations, we conclude that PPARγ causes activation of ERK1/2 and that activation of ERK1/2 is both necessary and sufficient for PPARγ-induced EMT. The mechanism whereby ERK1/2 promotes cellular motility has not been investigated in detail in intestinal epithelial cells, although ERK1/2 is known to play an essential role in regulating focal adhesion activity in other types of epithelial cells (24, 25). We observed that PPARγ promotes an increase in both the expression and the extent of Ser910 phosphorylation of FAK (Fig. 9C). Phosphorylation of FAK Ser910 was blocked by the MEK inhibitor U0126, indicating that PPARγ-mediated FAK phosphorylation occurs downstream of MEK activation. FAK Ser910 is a known ERK1/2 phosphorylation site and has been previously studied...
been shown to play a major role in activation of FAK and regulation of focal adhesion activity during cellular migration (26–28). These data suggest that PPARγ regulates motility, at least in part, by regulating cell-matrix interaction at the level of focal adhesions. However, other potential mechanisms may also be involved.

**PPARγ Activates Phosphoinositide 3-Kinase**—PPARγ promotes colony scattering via a mechanism that involves activation of ERK1/2 by a Cdc42/PAK1-dependent mechanism. Cdc42 is known to be activated by PI3K, and PPARγ induced the p110α catalytic subunit of PI3K (Fig. 10A), with no change in expression of the p85 regulatory subunit. PTEN expression was not affected by PPARγ. However, we observed an increase in phosphorylation of Akt1, indicating that the observed increase in PI3K expression is associated with a corresponding increase in PI3K activity. Conversely, we observed no change in expression of p110α or phosphorylation of Akt1 when the PPARγ agonist was added to RIE/S3 cells, demonstrating that PI3K activation requires PPARγ.

These observations suggest that PPARγ activates PI3K, which may in turn activate Cdc42/PAK1. To test this hypothesis, we measured the effects of PI3K inhibitors upon phosphorylation of Ser298 of MEK1. The PI3K inhibitor LY294002 blocked PAK-dependent phosphorylation of MEK1 Ser298 as well as activation of ERK1/2 by PPARγ (Fig. 10B). LY204002 blocked PPARγ-dependent cytodifferentiation of RIE/S3 cell colonies (Fig. 10C). However, LY294002 had no effect on the mesenchymal phenotype invoked by constitutively active MEK1 or MEK2. These data indicate that PI3K is required for PPARγ-mediated colony scattering and is upstream of ERK1/2 in the PPARγ-mediated morphogenetic pathway.

**DISCUSSION**

Although there are several reports of the effects of steroid receptors on the motility and invasiveness of tumor cells of various sorts, little is known about the role...
FIGURE 10. RS5444 induces PI3-kinase. A, RIE/S3γ1 cells were incubated with Me₃SO or RS5444 for 24 h. Cell lysates were analyzed by Western blotting with antibodies to p110α, p85, PTEN, phospho-AKT T423, total AKT, and actin. B, RIE/S3γ1 cells were incubated with Me₃SO or RS5444 in the presence or absence of 50 μM LY294002 for 24 h. Cell lysates were analyzed by Western blotting using antibodies to phospho-MEK1 (Ser298), total MEK1, phospho-ERK1/2, and total ERK1/2. C, RIE/S3γ1 cells stably expressing empty vector pLNCX were incubated with 50 μM LY294002 alone, or LY294002 plus RS5444 for 24 h. RIE/S3γ1 cells stably expressing constitutively active MEK1 or MEK2 were incubated with Me₃SO or LY294002 for 24 h prior to staining. The bars equal 20 μm. DMSO, dimethyl sulfoxide.
activation to migration initiates. We have previously shown that PPARγ inhibits proliferation and induces EMT of intestinal epithelial cells (11). Our data indicate that PPARγ activates ERK1/2 by a mechanism that requires PI3K and MEK1/2 and involves the Rho family member Cdc42 and its downstream effector PAK. To our knowledge, no such pathway has ever been described for a nuclear receptor. It is known that some steroid hormone receptors activate MAPK signaling by a mechanism that does not appear to involve transcriptional regulation. These so-called nongenomic effects of the progesterone and estrogen receptor are rapid and transitory, do not require DNA binding activity, and are generally thought to be involved in a feedback loop that controls receptor activity (29–31). PPARγ-mediated activation of ERK1/2 is delayed and persistent, and PPARγ-mediated EMT does not occur in cells that express the DNA-binding domain mutant C129S receptor. Our data are consistent with the hypothesis that MAPK activation results directly from induction of PI3K. However, the activity of PI3K is regulated by a large number of mechanisms, and additional experiments will be required to define the mechanism of activation upstream of PI3K.

We introduced these studies by referring to a series of experiments suggesting that whereas PPARγ is, in general, a tumor suppressor, thiazolidinediones may, in certain genetic backgrounds, promote tumorigenesis. In this regard, the parallel between the effects of PPARγ and TGF-β on intestinal epithelial cells is provocative. Like PPARγ, both TGF-β and the TGF-β type II receptor are induced at the crypt/villus junction (32, 33), and both TGF-β and PPARγ inhibit cell cycle progression of intestinal epithelial cells (34). TGF-β activates Rho family GTPases (35, 36), induces EMT, and stimulates RIE cell motility (37). Like PPARγ, TGF-β appears to act both as a suppressor and a promoter of tumorigenesis (38, 39). Our present understanding of this dual nature of TGF-β suggests that the tumor-suppressive effects derive from inhibition of cell proliferation, whereas the tumor-promotive effects appear to be related to the ability of TGF-β to promote EMT. It is tempting to extend this analogy and to speculate that the tumor suppressive effects of PPARγ may be due to inhibition of proliferation, whereas the ability of PPARγ to promote tumorigenesis under certain circumstances may be related to the ability of this receptor to regulate processes that are involved in cellular motility and adhesion. Confirmation of this hypothesis will obviously require additional experiments to dissect the effects of PPARγ on proliferation and motility/adhesion in tumor cells.

A major focus of our investigation has been to define the physiological roles of PPARγ in intestinal epithelial cells. It has long been suspected that PPARγ regulates some aspect of the differentiated phenotype of intestinal epithelial cells, and our studies elucidate a heretofore unsuspected role of this receptor in regulating cell motility, a very important differentiated function of villus epithelial cells. We have previously shown that PPARγ also inhibits cellular adhesion (11), and it may be that one of the roles of this receptor is to regulate exfoliation from the villus tips. The mechanism that accounts for changes in cellular adhesion remains to be investigated, but one obvious question relates to the role of PI3K and Cdc42 in this process. It should also be pointed out that most of our experiments have utilized pharmaceutical agonists, which raises the question of the extent to which similar processes may be invoked by natural ligands. The nature of the endogenous ligands that regulate PPARγ activity in vivo is poorly defined. Although the naturally occurring prostanoid agonist (15d-D12,14-prostaglandin J2) clearly promotes EMT, it is not certain that this ligand plays an important role in regulating PPARγ in epithelial cells. PPARγ is activated by a number of polyunsaturated long chain fatty acids, including essential dietary fatty acids such as eicosohexaenoic acid (1, 40, 41), and it is tempting to speculate that one possible role of PPARγ may be to influence the rate of renewal of the intestinal epithelium in response to dietary lipids. Homozygous knock-out of PPARγ in colonic epithelial cells has recently been described (42), and experiments are currently underway to determine whether PPARγ knock-out in the intestinal epithelium affects renewal of the epithelium or the response of intestinal epithelial cells to naturally occurring PPARγ agonists in vivo. Elucidation of the function of PPARγ in intestinal epithelial cells will provide new understanding of the physiological role of this receptor in vivo and may provide new insight into the role of PPARγ in carcinogenesis.

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