Annexin 2 Regulates Intestinal Epithelial Cell Spreading and Wound Closure through Rho-Related Signaling

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Epithelial cell migration is a critical event in gastrointestinal mucosal wound healing and is dependent on actin cytoskeletal reorganization. We observed increased expression of an actin regulatory protein, annexin 2, in migrating intestinal epithelial cells. Small interfering RNA (siRNA)-mediated knockdown of annexin 2 expression in Caco-2 epithelial cells resulted in significant reductions in cell spreading and wound closure associated with decreased formation of filamentous actin bundles along the base of migrating cells. Because annexin 2 has been shown to influence actin cytoskeletal remodeling through targeting signaling molecules to membrane domains, we examined the membrane association and activation status of Rho GTPases after annexin 2 knockdown. We observed Rho dissociation from membranes and decreased Rho activity following annexin 2 siRNA transfection. Inhibition of cell spreading and wound closure in annexin 2 siRNA-transfected cells was prevented by expression of constitutively active RhoA. Rho colocalized with annexin 2 in lamellipodia and along the cytoplasmic face of the plasma membrane. In addition, annexin 2 was observed to co-immunoprecipitate with endogenous Rho and constitutively active RhoA. These findings suggest that annexin 2 plays a role in targeting Rho to cellular membranes, thereby modulating Rho-related signaling events regulating cytoskeletal reorganization during epithelial cell migration. (*Am J Pathol* 2007, 170:951–966; DOI: 10.2353/ajpath.2007.060647)

The epithelial lining of the gastrointestinal tract forms a selectively permeable barrier separating luminal content from underlying tissues.^{1,2} Surface and crypt epithelial

damage occurs in many pathological processes including infectious colitis and inflammatory bowel disease. Breakdown of this barrier results in fluid and electrolyte loss as well as an influx of antigens, which further exacerbate inflammatory responses.³ To re-establish epithelial barrier function, the epithelium must efficiently reseal the mucosal defects. A major mechanism by which wound healing is achieved involves epithelial cell migration also referred to as “restitution.”^{4,5}

A central event in the cell migratory process is active reorganization of filamentous actin. As cells become motile, they extrude actin-rich projections (lamellipodia/filopodia) that transiently adhere to the underlying matrix to create traction forces necessary for forward cell movement.^{4–6} Orchestrated F-actin restructuring requires interaction of numerous actin binding and regulatory proteins with the actin cytoskeleton.

Annexin 2 is a calcium-dependent phospholipid binding protein that also associates with actin filaments⁷ and mediates membrane-membrane and membrane-cytoskeletal interactions. Thus it plays an important role in membrane trafficking and stabilization of membrane-associated protein complexes with the actin cytoskeleton.^{7–11} Annexin 2 also plays a role in regulating the actin cytoskeleton and has been implicated in cell migration.^{7,11,12} Studies using Moloney sarcoma virus-transformed Madin-Darby canine kidney (MDCK) cells and Lewis lung carcinoma cell lines suggest that annexin 2 suppresses cell motility.^{13,14} Other studies suggest that surface annexin 2 positively regulates migration through interactions with an extracellular matrix protein, tenascin-C.^{15,16} In addition, invasive neoplasms such as ovarian and renal cell carcinoma show increased annexin 2 ex-

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pression, whereas others such as prostate cancer lose its expression.^{17–20} Thus, the role of annexin 2 in epithelial cell migration is unclear and remains to be elucidated.

We identified up-regulation of annexin 2 expression during migration of two model intestinal epithelial cell lines, T84 and Caco-2. The present study was therefore designed to investigate the role of annexin 2 in intestinal epithelial cell migration. Using a gene silencing approach with small interfering RNA (siRNA),²¹ we provide evidence suggesting that annexin 2 regulates Rho-membrane interactions that impact downstream signaling pathways resulting in alterations in F-actin networks and an inhibition of Caco-2 cell motility.

Materials and Methods

Cell Culture

Human intestinal epithelial cell lines (T84 and Caco-2) were used in these studies. T84 cells were used for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis of annexin 2 expression. Because Caco-2 cells are amenable to transfection and showed similar changes in annexin 2 expression after wounding compared with that in T84 cells, they were used for functional analysis and biochemical studies (see below). Cells were passaged and seeded on collagen-coated permeable supports or tissue culture-treated plates (Costar, Cambridge, MA). T84 cells were grown in 1:1 Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 14 mmol/L NaHCO₃, L-glutamine, 40 μ g of penicillin, 8 μ g/ml ampicillin, 90 μ g/ml streptomycin, and 6% fetal bovine serum (FBS) as previously described.²² Caco-2 cells were grown in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 15 mmol/L HEPES, pH 7.4, 2 mmol/L L-glutamine, and 1% nonessential amino acids.²³

cDNA Microarray and RT-PCR Analysis

T84 cells were grown to confluence on 45-cm² collagen-coated permeable supports. Multiple parallel wounds were created mechanically using a specialized wounding comb as previously described.²⁴ Wounded and non-wounded monolayers were then incubated for 6 hours. For microarray analysis, RNA extraction was performed using Trizol LS (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions followed by DNase digestion (Promega, Madison, WI). mRNA was purified using the Oligotex system (QIAGEN, Valencia, CA) and sent to Incyte Genomics (Wilmington, DE) for microarray analysis. For RT-PCR, RNA was extracted using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed with an RT-Advantage kit (BD Biosciences) using hexamer primers. Standard PCR was performed on cDNA to control for genomic contamination and other nonspecific products. The following primers were used:

forward, 5'-AGATCATCTGCTCCAGAACCAACC-3'; reverse, 5'-GGGACTTCGCGTACTTTCTCTTGA-3'. Real-time quantitation was performed using the iCycler system (Bio-Rad, Hercules, CA) as previously described.²⁵ In brief, PCR amplification reactions were performed using buffer containing SYBR Green (Applied Biosystems, Foster City, CA). Positive control reactions were performed to determine the linear range of detection and establish a standard curve for each transcript. Unknowns were amplified, and cDNA was diluted to produce threshold values within the linear range of detection. Transcripts were then quantified from the corresponding standard curve, with β -actin as an internal control.

Immunoblot Analysis

Confluent T84 and Caco-2 monolayers were grown on collagen-coated 5-cm² permeable supports and wounded using a specialized wounding comb that essentially converts the entire monolayer into spreading and migrating cells.²⁴ Medium was changed after wounding, and control monolayers were subject to medium change only. Cells were incubated over a 3-day time course. Cells were harvested in Hanks' balanced salt solution (HBSS+) containing protease and phosphatase inhibitor cocktails (Sigma Chemical Co., St. Louis, MO) and nitrogen-cavitated (200 psi, 15 minutes). Post-nuclear supernatants (1000 $\times$ g, 5 minutes centrifugation) were normalized for protein concentration using a bicinchoninic acid assay (Pierce, Rockford, IL) and subjected to Western blot analysis. Membrane preparations were made by centrifugation of postnuclear supernatants (see above) at 170,000 $\times$ g for 45 minutes. Supernatants (cytosolic fraction) were collected, and the pellet was resuspended in HBSS+ containing 1% *n*-octylglucoside by sonication on ice (15 pulses, 10% duty cycle, 10% output). Fractions were normalized for protein concentration and subject to Western blot analysis. Densitometric analysis was performed using the UN-SCAN-IT automated digitizing system (Silk Scientific, Orem, UT).

siRNA Transfections

Control siRNA (scramble duplex) and a siRNA duplex targeting nucleotides 94 to 113 of annexin 2 mRNA²⁶ were obtained from Dharmacon (Lafayette, CO). Transfections were performed using Lipofectamine 2000 in Opti-MEM I medium (Invitrogen, Carlsbad, CA). Lipofectamine 2000 and siRNA (20 μ mol/L stock) were diluted separately in Opti-MEM I at a ratio of 1:25 and incubated at room temperature for 5 minutes. Equal volumes of siRNA and Lipofectamine 2000 solutions were mixed and incubated at room temperature for 15 minutes. Subconfluent Caco-2 monolayers were washed and placed in Opti-MEM I media. Transfection solutions were diluted 1:5 into cultures for a final siRNA concentration of 80 nmol/L. After incubation overnight, the monolayers were placed into complete media.

Recombinant Adenoviral Vectors and Adenoviral Infection

A recombinant adenoviral vector encoding a 6x myc-tagged siRNA-resistant annexin 2 mutant was generated for rescue studies. Five silent mutations were introduced into the siRNA target sequence by PCR amplification of template annexin 2 using the following primers: forward 5'-CGCGGATCCACCATGTCTACTGTTCACGAAATCCTGTGCAAGCTCAGCTTGGAGGGTGACCACAGCACACCGCCAAGTGCAT-3'; reverse 5'-TCGCGGATCCGTCACTCTCCACCACACAGGTAC-3'. The amplicon was then ligated into pcDNA3_6xMyc vector using *Bam*HI.²⁷ The myc-tagged siRNA-resistant annexin 2 sequence was then subcloned into pShuttle CMV (Stratagene, La Jolla, CA) using *Kpn*I and *Not*I and confirmed through sequencing. Adenoviral production was performed using the AdEasy adenoviral vector system according to the manufacturer's protocol (Stratagene). A recombinant adenoviral vector encoding N-terminal myc-tagged, constitutively active RhoA (myc-RhoAV14) was a generous gift from James Bamberg (Colorado State University).²⁸ Adenoviral transfections were performed the day following siRNA transfection by incubating cells in low calcium medium [calcium-free Eagle's minimum essential medium with 10% dialyzed FBS, containing <10 μ mol/L extracellular Ca^{2+}] with viral particles diluted to 5×10^5 plaque-forming units/ml. Cells were then placed back in complete media and incubated for 2 days before analysis.

Generation of a Stable Inducible Caco-2 Cell Line Expressing Enhanced Green Fluorescent Protein (EGFP)-Tagged RhoAV14

The GeneSwitch system (Invitrogen) was used to generate a mifepristone-inducible Caco-2 cell line expressing EGFP-tagged RhoAV14. The RhoAV14 mutant sequence was amplified from a pU-myc-RhoA-V14 construct (gift of M. Symons, Picower Institute, Manhasset, NY) using the following primers: forward, 5'-CGGAATTCAATGGCTGCATCCGGAAGAAACTG-3'; reverse, 5'-GCGGATCCGGACAAGACAAGGCAACCAG-3'. Amplified sequences were digested with *Eco*RI 5' and *Bam*HI and ligated into pEGFP N3 plasmid (BD Biosciences). The fusion sequence was subcloned into pGene/V5-His B using *Eco*RI and *Not*I. Plasmids were sequenced for confirmation. The regulatory plasmid and RhoAV14-EGFP expression vector were transfected into Caco-2 cells and selected in media containing hygromycin (300 μ g/ml) and zeocin (200 μ g/ml). Induction of RhoAV14-EGFP expression was achieved by incubation of cells in complete media containing 1×10^{-8} mol/L mifepristone.

Antibodies, Immunofluorescence, and Image Analysis

Monoclonal anti-annexin 2, mouse monoclonal anti-occludin, and rabbit polyclonal anti-JAM-A antibodies were

obtained from Zymed (San Francisco, CA); mouse monoclonal anti-Rho (-A, -B, -C) antibodies from Upstate Biotechnology (Charlottesville, VA); and goat anti-annexin 8 antibodies, rabbit polyclonal anti-RhoA (119), and anti-actin antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-RhoA (119) cross-reacts with RhoC and to a lesser extent RhoB as per the manufacturer. Mouse anti-annexin 1 and anti-annexin 6 antibodies were obtained through BD Biosciences. Mouse monoclonal and rabbit polyclonal anti-GFP antibodies were obtained from Sigma Chemical Co. Alexa Fluor 488- or 546-conjugated secondary antibodies, Alexa Fluor 488-conjugated phalloidin, and Topro-3 iodide were obtained from Invitrogen.

Immunofluorescence studies were performed on cells grown on 0.33-cm² polycarbonate collagen-coated permeable supports. Cells were fixed/permeabilized in 100% ethanol for 20 minutes at -20°C . All subsequent steps were performed at room temperature. Cells were washed with HBSS+ and blocked in HBSS+ with 3% bovine serum albumin (BSA) for 1 hour. Primary antibody reactions were performed in HBSS+ with 3% BSA for 1 hour (1:200 dilution for anti-annexin 2 antibody). Secondary antibodies and Alexa Fluor 488-conjugated phalloidin were diluted 1:1000 in 3% BSA and incubated with monolayers for 45 minutes. Topro-3 iodide was diluted 1:1000 in HBSS+ and added to monolayers for 5 minutes after secondary reactions. Monolayers were then washed and mounted. For annexin 2 and Rho colocalization studies, cells were washed in HBSS+ and cooled to 4°C . Cells were then permeabilized at 4°C for 20 minutes using 0.05% saponin in HBSS+. Cells were then washed, blocked with 3% BSA in HBSS+ for 1 hour, and incubated with rabbit polyclonal anti-RhoA (119) antibodies (1:100 dilution; Santa Cruz Biotechnology, Inc.). Monolayers were washed, fixed with 100% methanol (15 minutes at -20°C), and then incubated with anti-annexin 2 antibodies and secondary antibodies as above.

Confocal microscopy was performed using the Zeiss LSM 510 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). For pixel intensity analysis, actin and annexin 2 staining was performed on monolayers 4 days after siRNA transfection as above. A total of five areas per experimental group were imaged all with the same contrast and detector gain settings for each channel. The average percentages of maximum pixel intensities were obtained using AxioVision software (Release 4.3; Carl Zeiss Microimaging, Inc.).

Cell Spreading Assay

Four days after siRNA transfection and 3 days after adenoviral transfection, Caco-2 cells were trypsinized and washed with HBSS+ containing 0.1% BSA. Cells were suspended in HBSS + 0.1% BSA and counted, and equal numbers (10,000 cells/well) were plated in collagen IV-coated, collagen I-coated (5 μ g/ml; BD Biosciences), or laminin-coated (Chemicon International, Temecula, CA) 96-well plates that had been blocked with 0.1% BSA for 1 hour. Cells were allowed to adhere and

spread for 2 hours at 37°C. Images were taken using a Zeiss Axiovert microscope with an attached charge-coupled device (CCD) camera under differential interference contrast and fluorescence microscopy. Cells were counted manually, and a total of 10 fields were counted per group in each experiment. Cells that demonstrated round shape with a distinct and defined edge surrounding the entire circumference of the cell were defined as "nonspreading." Cells exhibiting visible lamellipodial/filopodial extrusion or polygonal shape were defined as "spreading." The percentage of spreading cells was calculated as the number spreading cells divided by the total number of cells in that field.

Restitution Assay

Caco-2 cells grown in 24-well culture plates were transfected at ~65% confluency with siRNA. Adenoviral transfections were performed 24 hours after siRNA transfection as described above. Four days after siRNA transfection, when cells reached 100% confluency, a single linear wound was created through the monolayer with a sterile pipette tip. Sites at which wounds were to be measured were marked on the undersurface of the wells to ensure that measurements were taken at the same place. Wounds were imaged at 0 and 16 hours on a Zeiss Axiovert microscope with an attached CCD camera. Wound widths were measured from the images using Scion Image software (Scion Image Corp., Frederick, MD). Ten measurements along the wound length were averaged to determine the wound width.

Monomeric/Filamentous Actin Fractionation

Monomeric and filamentous actin pools were fractionated as previously described.²⁹ In brief, 4 days after transfection, Caco-2 monolayers were treated with extraction buffer containing 1% Triton X-100, 2 μ g/ml phalloidin, and protease and phosphatase inhibitor cocktails (Sigma Chemical Co.) after three washes with HBSS+. The Triton X-100 soluble fraction was subjected to centrifugation (16,000 $\times g$ for 20 minutes) to remove insoluble debris. The monolayers were washed, and the Triton X-100 insoluble fraction was collected using a second buffer [20 mmol/L Tris HCl, 150 mmol/L NaCl, 2 mmol/L ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.2 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 1% sodium deoxycholate, pH 7.4] with protease and phosphatase inhibitors. These fractions were then homogenized using a borosilicate douncer (20 dounces each; Wheaton, Millville, NJ). Equal volumes of each fraction were added to an equivalent volume of 2 $\times$ SDS sample buffer and subjected to Western blot analysis. Total actin levels were determined from whole cell lysates normalized for protein concentration.

Rho Activation Assay

To determine Rho activity, a commercially available activation assay was used (Upstate Biotechnology). In brief,

monolayers were washed in cooled Tris-buffered saline before lysis in supplied magnesium lysis buffer (MLB) buffer containing protease inhibitors. Lysates were incubated at 4°C with gentle agitation before centrifugation (14,000 $\times g$ for 5 minutes). Supernatants were normalized for protein concentrations and incubated with recombinant Rhotekin-glutathione S-transferase coupled to agarose beads (45 minutes, 4°C with rotation). Positive control lysates were loaded with guanosine 5'-O-(3-thio)triphosphate before incubation with Rhotekin-conjugated beads. Beads were washed with MLB buffer and resuspended in SDS sample buffer for Western blot analysis using anti-Rho (-A, -B, -C) antibodies.

Immunoprecipitation

Monolayers were washed and lysates harvested in lysis buffer (1% Triton X-100, 0.5% Igepal, 150 mmol/L NaCl, 1 mmol/L EGTA, pH 8.0, 1 mmol/L EDTA, 0.2 mmol/L sodium orthovanadate, 10 mmol/L Tris, pH 7.4, and 20 mmol/L imidazole) with protease inhibitor cocktail. Protein A- or protein G-coupled Sepharose beads (Amersham Biosciences, Buckinghamshire, UK) were used for these experiments. Centrifugation at 800 $\times g$ for 5 minutes was performed to pellet beads. The beads were washed with lysis buffer at 4°C for 30 minutes. Lysates were precleared with a 40- μ l slurry of washed beads at 4°C for 1 hour. Lysates were then incubated with 5 μ g of anti-RhoA (119) antibody (Santa Cruz Biotechnology), control rabbit IgG (Lampire Biological Laboratories, Pipersville, PA), or anti-EGFP antibody (Sigma Chemical Co.) for 3 hours (4°C with rotation). Lysate-antibody solutions were incubated with 50 μ l of washed beads at 4°C for 3 hours. Beads were pelleted and washed three times (10 minutes/wash at 4°C) before the addition of SDS sample buffer. Beads were then boiled for 5 minutes and pelleted (14,000 $\times g$ for 5 minutes). Supernatants were loaded into a single well for Western blot analysis. Membranes were sequentially immunoblotted using anti-Rho or anti-EGFP antibodies followed by anti-annexin 2 antibodies.

Statistics

Experiments were performed independently at least three times. Results are expressed as the mean $\pm$ SEM. Paired Student's *t*-tests were used to compare results from different trials.

Results

Annexin 2 Expression Is Up-Regulated in Migrating Intestinal Epithelial Cells

To gain insight into the molecular mechanisms underlying epithelial cell migration, we compared gene expression profiles of stationary and migrating T84 cells using cDNA microarray analysis. Polarized epithelial cells grown as a

Figure 1. Annexin 2 expression is up-regulated in migrating intestinal epithelial cells. Confluent T84 monolayers were wounded and allowed to migrate for 6 hours. Annexin 2 mRNA levels were compared in migrating and stationary cells by RT-PCR (**A** and **B**). A twofold increase in annexin 2 mRNA transcripts was observed in migrating cells relative to stationary cells (* $P < 0.05$). Annexin 2 protein expression was examined in migrating and stationary T84 and Caco-2 cells by Western blotting (**C**). Actin was used as a loading control. Densitometry of Western blots is shown in **D**. Annexin 2 protein levels were increased ~2.5- and 1.8-fold in migrating T84 and Caco-2 cells, respectively, during the first 24 hours of migration.

monolayer on permeable supports were designated as stationary cells. To generate migrating cells, we induced multiple wounds in the monolayer to convert the monolayer into motile epithelial cells. RNA isolated from stationary cells versus cells migrating for 6 hours were submitted to Incyte Genomics for microarray analysis. A total of 16 genes showed differential expression by a factor of twofold or more. Among the 11 up-regulated genes were keratins 8 and 18, matrix metalloproteinase 9, epithelial membrane protein 1, cytokeratin 20, annexin 1, and annexin 2 (data not shown). Because annexin 2 has been shown to play an important yet undefined role in cell migration,^{14,15} and annexin 2 transcripts were up-regulated 2.5-fold in migrating cells (data not shown), experiments were performed to understand its role in the migration of intestinal epithelial cells.

RT-PCR followed by real-time PCR was performed to confirm our microarray results. Consistent with the microarray data, annexin 2 mRNA levels were twofold higher in epithelial cells migrating for 6 hours when compared with polarized cells in nonwounded monolayers (Figure 1, A and B; * $P < 0.05$). Comparison of annexin 2 protein levels in the above migrating and stationary cells revealed that the annexin 2 protein levels were significantly increased as early as 2 hours after wounding in T84 and another model intestinal epithelial cell line, Caco-2, and remained elevated over the first 24 hours (Figure 1C). Analogous to the mRNA increase, densitometric analysis of Western blots demonstrated, on average, a 2.2-fold increase in annexin 2 protein levels in T84 cells and 1.8-fold increase in Caco-2 cells (Figure 1D). The increase in annexin 2 protein expression was transient and subsequently returned to that of stationary cells by 48 hours (data not shown).

Functional Knockdown of Annexin 2 Inhibits Caco-2 Cell Spreading and Wound Closure

Given that annexin 2 is up-regulated in migrating cells, we tested the effects of functional knockdown of annexin 2 by RNA interference on intestinal epithelial cell motility.²¹ T84 cells were not amenable to transfection and therefore our subsequent studies were undertaken using Caco-2 cells. A previously described siRNA duplex targeting annexin 2 transcripts was used²⁶ along with a scramble as control. An siRNA-resistant 6x myc-tagged annexin 2 construct (AnxA2Ri-6xmyc) was used to demonstrate the specificity of the effects of knockdown of endogenous annexin 2 expression. Western blots of Caco-2 cells 4 days after transfection with annexin 2 siRNA, and 3 days after adenoviral infection, demonstrated significant and specific reduction in endogenous annexin 2 protein levels compared with controls (Figure 2A). The scramble duplex did not affect annexin 2 protein levels and no changes in the expression of other representative annexin family members (annexin 1, 6, and 8) were observed. In addition, expression of AnxA2Ri-6xmyc was confirmed in both scramble and annexin 2 siRNA-transfected cells. Endogenous annexin 2 protein levels were reduced by 75% to that of controls based on densitometric analysis (Figure 2B). Furthermore, we did not observe any effects of annexin 2 knockdown on cell viability or proliferation as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assays and cell counts before and after transfection (data not shown).

To analyze the role of annexin 2 in epithelial cell motility, we first examined the ability of Caco-2 cells to spread on extracellular matrix proteins. Epithelial cells

Figure 2. siRNA-mediated knockdown of annexin 2 expression. Caco-2 cell monolayers were transfected with scramble control or annexin 2 siRNA with or without subsequent transfection of an adenoviral vector encoding an siRNA-resistant annexin 2 construct (AnxA2Ri-6xmyc). Analysis of endogenous annexin 2 and AnxA2Ri-6xmyc expression was determined by Western blotting (**A**) 4 days after siRNA transfection and 3 days after adenoviral infection. Western blot analysis showed significant decrease in endogenous annexin 2 expression in annexin 2 siRNA-transfected cells but not in scramble control siRNA-transfected cells (**A**). Actin was used as a loading control, and no changes in other representative annexin family members (1, 6, and 8) were observed. From this analysis, a similar degree of expression of AnxA2Ri-6xmyc was also confirmed in both control and annexin 2 siRNA-transfected cells. Densitometric analysis revealed an average reduction in endogenous annexin 2 protein levels by 75% compared with controls (**B**).

associate with a variety of extracellular matrix proteins including collagens, fibronectin, and laminin. We analyzed cell spreading on collagen I, collagen IV, and laminin. We found that Caco-2 cells spread poorly on fibronectin and therefore did not pursue studies with this matrix protein. Four days after transfection with the respective siRNAs, and 3 days after AnxA2Ri-6xmyc adenoviral transfection, cells were harvested by trypsinization, washed, and allowed to spread on collagen I-coated plates for 2 hours at 37°C. As shown in the representative differential interference contrast images of cell spreading in Figure 3A, we observed a reduction in the number of

spreading cells when annexin 2 expression was down-regulated with siRNA. The number of spreading cells was restored to that of controls in annexin 2 siRNA-transfected cells expressing AnxA2Ri-6xmyc. On average, the annexin 2 siRNA-transfected group exhibited a 45 to 50% reduction in cell spreading compared with controls and was rescued by expression of AnxA2Ri-6xmyc (Figure 3B; $*P < 0.05$). Similar inhibition of cell spreading due to annexin 2 knockdown was observed on collagen IV and laminin (data not shown). Thus, annexin 2 knockdown significantly reduced Caco-2 cell spreading. The extent of individual cell spreading, or surface area covered by individual cells, was also assessed using AxioVision software (Zeiss). In all groups, individual spreading cells showed variable amounts of covered surface area (ranging from ~20 to 100 μm^2), and thus no statistically significant difference was observed for this parameter (data not shown).

We next performed restitution assays to further examine the role of annexin 2 in epithelial cell migration. Four days after siRNA transfection and 3 days after adenoviral transfection, mechanical wounds were introduced into confluent monolayers. Migration of epithelial cells into wounded areas was analyzed. Wound closure was determined using microscopy and image analysis. Wound widths were measured over a period of 16 hours, and the percentage of wound closure was determined. Wound closure at 16 hours was reduced by 40% in annexin 2 siRNA-transfected monolayers compared with controls (Figure 3, C and D; $*P < 0.05$). The expression of AnxA2Ri-6xmyc in annexin 2 siRNA-transfected cells restored the percentage of wound closure to that of controls. Thus, siRNA-mediated down-regulation of annexin 2 expression inhibited Caco-2 wound closure.

Annexin 2 Regulates F-Actin Networks in Migrating and Stationary Caco-2 Cells

Dynamic F-actin restructuring is required for the development of cell-matrix adhesions and cell migration.^{30,31} Because annexin 2 has been shown to regulate the actin cytoskeleton³² and knockdown of annexin 2 expression impaired Caco-2 cell migration, we analyzed the distribution of annexin 2 in relation to F-actin and examined the effects of annexin 2 knockdown on F-actin architecture. Previous studies have reported distribution of annexin 2 in the submembranous region of epithelial cells where it colocalizes with F-actin and within lamellipodial protrusions of migrating cells.^{7,33} Similar to these reports, we identified annexin 2 predominantly along the apical and lateral subcortical compartments of polarized Caco-2 epithelial cells where it colocalized with F-actin networks (Figure 4A, a–c). A similar pattern of staining was identified in polarized T84 cells (data not shown). In migrating Caco-2 cells, we identified annexin 2 within the cytoplasmic compartment of lamellipodial extrusion (Figure 4B, a–c). We did not, however, observe significant colocalization of annexin 2 with F-actin structures within lamellipodia (Figure 4B, a–c). We also examined annexin 2 localization in colonic mucosa. Frozen sections of normal

Figure 3. Annexin 2 knockdown inhibits Caco-2 cell spreading and wound closure 4 days after siRNA transfection and 3 days after adenoviral infection. Caco-2 cells were trypsinized and subjected to a spreading assay (**A** and **B**; data shown for collagen I). Representative differential interference contrast images of spreading on collagen I from these experiments (**A**) showed a prominent population of rounded cells in the annexin 2 siRNA-transfected group compared with nontransfected and scramble control siRNA-transfected cells. Annexin 2 knockdown resulted in a 47% reduction in cell spreading compared with controls (**B**, $*P < 0.05$). Expression of AnxA2Ri-6xmyc restored the number of spreading cells in the annexin 2 siRNA-transfected group compared with that of controls. AnxA2Ri-6xmyc expression in control siRNA-transfected cells did not significantly influence cell spreading. Nontransfected Caco-2 cell monolayers and those 4 days after siRNA transfection and, 3 days after adenoviral infection, were wounded and allowed to migrate in complete media for 16 hours. Percentage of wound closure was determined by image analysis (**C** and **D**). Monolayers transiently transfected with annexin 2 siRNA demonstrated a 40% reduction in wound closure as compared with controls ($*P < 0.05$), which was rescued by expression of AnxA2Ri-6xmyc.

mucosa were immunolabeled for annexin 2 and nuclei highlighted using Topro. Annexin 2 expression was identified in both crypt and surface enterocytes (Figure 4C, a and b). Annexin 2 was present along the lateral membrane domain (Figure 4C, c, arrows) consistent with its localization in Caco-2 and T84 cells. In addition, a pool of annexin 2 also appeared in the cytoplasmic compartment (Figure 4C, c, arrowheads).

Annexin 2 knockdown induced significant alterations in F-actin architecture that was visualized with Alexa-488 phalloidin staining. Although migrating cells with annexin 2 knockdown were able to extrude lamellipodia, they showed diminished density of F-actin bundles within lamellipodia and along the base of cells immediately behind the leading edge when compared with control-transfected cells [Figure 5A, a–d, L (lamellipodia) and

brackets]. To quantify these changes, multiple images from control and annexin 2 siRNA-transfected groups were taken with identical detector gain and contrast settings and average percentages of maximum pixel intensity for both annexin 2 and F-actin staining were obtained using AxioVision software (Zeiss). As shown in Figure 5B, the average percentage of maximum pixel intensity for actin staining in migrating cells with annexin 2 knockdown averaged 55% less than that of controls.

In stationary cells, annexin 2 knockdown altered columnar cell morphology. As shown in the reconstructed images in the XZ plane, cells lacking annexin 2 expression were more cuboidal than adjacent tall columnar nontransfected cells (Figure 5C). Similar findings were present in approximately 75% of areas showing annexin 2 knockdown in confluent monolayers and similar cuboi-

Figure 4. Annexin 2 localization in Caco-2 epithelial cells. Nonwounded Caco-2 cell monolayers were double-labeled for annexin 2 (red) and F-actin (green) (**A**). Annexin 2 expression was identified along the apical and lateral membrane domains (**A, a–c; XZ** images). Scale bar = 20 μ m. In migrating cells (**B**), annexin 2 was identified within lamellipodial extrusions and did not significantly colocalize with F-actin in these structures (**B, a–c**). Scale bar = 10 μ m. In frozen sections of normal colonic mucosa (**C**), annexin 2 expression was identified along the lateral membrane and within the cytoplasm of both crypt and surface enterocytes (**C, a and b**; scale bar = 50 μ m; **c and d**; scale bar = 20 μ m).

dal cells were not identified in control-transfected cells (data not shown). Interestingly, despite the decrease in cell height induced by annexin 2 knockdown, no changes in transepithelial resistance or the localization of the representative tight junction proteins ZO-1 and occludin were observed in annexin 2 siRNA-transfected monolayers (data not shown).

To detect dysregulation of actin polymerization and support our morphological findings, we used a biochemical approach with a Triton X-100 extraction assay to

examine the ratio of monomeric (G-actin) to F-actin. Four days following transfection with the respective siRNAs, extraction of monomeric actin (G-actin) was performed using 1% Triton X-100 (TX-100) in HBSS+. These studies were performed in both subconfluent/spreading Caco-2 monolayers as well as those 6 hours after wounding, which showed similar results. As seen in Figure 6A, annexin 2 knockdown induced an increase in TX-100-soluble actin relative to the insoluble pool, indicating an increased content of monomeric actin. Total actin

Figure 5. Annexin 2 regulates F-actin in migrating and stationary cells. Wounded *Caco-2* monolayers were double-labeled for annexin 2 (red) and F-actin 4 days after siRNA transfection (**A**). Annexin 2 knockdown decreased F-actin bundles along the base of lamellipodial extrusions and in cells immediately behind the leading edge compared with controls [**A**, **a–d**; L (lamellipodia), brackets]. Scale bar = 10 μ m. The average percentage of maximum pixel intensity for annexin 2 (red channel) and F-actin (green channel) is shown in **B** ($P < 0.05$). In stationary cells (**C**), knockdown of annexin 2 expression resulted in a more cuboidal morphology compared with adjacent nontransfected cells. Scale bar = 20 μ m.

expression remained unchanged, consistent with previous experiments. Densitometric analysis of Western blots showed that the average G-/F-actin ratios were 2:1 and 1:1 in annexin 2 knockdown and scramble control siRNA-transfected monolayers, respectively (Figure 6B). Taken together, the morphological and biochemical changes in F-actin architecture and polymerization suggest that annexin 2 regulates F-actin in *Caco-2* cells and are consistent with other studies implicating a role for annexin 2 in regulating the actin cytoskeleton.^{32,34}

Figure 6. Annexin 2 knockdown decreases F-actin content in *Caco-2* cells. *Caco-2* cells were transfected with scramble or annexin 2 siRNA. Triton X-100 soluble and insoluble pools of actin were analyzed by Western blotting. Representative Western blot (**A**) and densitometric analysis of blots from three independent experiments (**B**) revealed significant increase in TX-100-soluble actin (G-actin) in annexin 2 siRNA-transfected cells with no changes in total actin.

*Annexin 2 Knockdown Results in Reduced Membrane Association and Activation of Rho in *Caco-2* Epithelial Cells*

Rho GTPases play a central role in the regulation of F-actin networks in various cell types, including epithelial cells. Studies suggest that annexin 2 regulates actin polymerization through targeting signaling molecules to membrane domains.¹² Because down-regulation of annexin 2 expression significantly influenced F-actin organization, we sought to examine the effects of annexin 2 knockdown on the membrane association and activation of Rho GTPases. We focused on Rac1, because it regulates the formation of branched F-actin networks within lamellipodia, and Rho, which is required for stress fiber formation and actin-myosin contractile events.^{35,36} These studies were also performed in both subconfluent/spreading *Caco-2* cell monolayers and those wounded and allowed to migrate for 6 hours. Cells were harvested, and membrane and cytosolic fractions were analyzed for Rho/Rac1 content by Western blotting. The Rho antibodies used in these experiments do not definitively distinguish among RhoA, -B, or -C, and thus the term Rho is used in describing these results. Although total levels of Rho were not affected, annexin 2 siRNA-transfected cells demonstrated reduced Rho content in membrane fractions as compared with scramble control siRNA-transfected cells with a concomitant increase in the cytoplasmic pool of Rho (Figure 7A). As a control for these studies, we examined these fractions for JAM-A and occludin content, which were present in only the membrane fractions, as expected (Figure 7A). Densitometric analysis revealed a 50% reduction in membrane-associated Rho in monolayers of cells that had been transfected with annexin 2 siRNA (Figure 7B). No changes in total or membrane-associated Rac1 were identified due to annexin 2 knockdown (Figure 7, A and B). We next exam-

Figure 7. Annexin 2 knockdown decreased the membrane association and activation of Rho. The membrane association and activation of Rho was determined in Caco-2 monolayers 4 days after transfection with scramble control or annexin 2 siRNA. Representative blots (**A**) and densitometric analysis (**B**) revealed significant reduction in the membrane to cytosolic ratio of Rho in annexin 2 siRNA-transfected cells. Immunoblotting for JAM-A and occludin were performed on the fractions as a control (**A**). Transfection with annexin 2 siRNA also reduced the levels of activated Rho (**C** and **D**). No changes in the membrane association or activation of Rac1 were observed following annexin 2 knockdown (**A–D**).

ined the activation status of Rho/Rac 1 under the above experimental conditions. Although the amount of active Rho in controls varied from ~10 to 20% of the total Rho, annexin 2 siRNA-transfected cells showed consistent reductions in the levels of active Rho (Figure 7, C and D). Due to this variability between assays, the densitometric analysis of Western blots from these studies is shown in Figure 7D as the average ratios of total and active Rho in annexin 2 and control siRNA-transfected cells. The average ratio of total Rho was 0.98, indicating no significant changes in total Rho consistent with results shown in Figure 7A. However, the ratio of active Rho in annexin 2 siRNA-transfected cells over control-transfected cells was 0.62, indicating ~40% reduction in its activation due to annexin 2 knockdown. No significant alterations in Rac1 activation status were detected (Figure 7, C and D).

The data shown in Figure 7 are derived using wounded monolayers. Similar results were obtained using subconfluent monolayers (data not shown).

Expression of Constitutively Active RhoA (RhoAV14) Prevents the Spreading and Wound Closure Defects Induced by Annexin 2 Knockdown

To confirm our findings suggesting that the effects of annexin 2 knockdown are mediated through decreased Rho-dependent signaling, we examined whether expression of constitutively active RhoA could reverse the effects of annexin 2 knockdown on cell spreading and

Figure 8. Expression of constitutively active RhoA (RhoAV14) prevents the impairment of cell spreading and wound closure induced by annexin 2 knockdown. Caco-2 cells were infected with an adenoviral vector encoding myc-RhoAV14 + EGFP the day after siRNA transfection and were subjected to spreading assays 3 days later. Representative differential interference contrast and fluorescence images (**A**) show cell spreading and myc-RhoAV14 + EGFP expression in scramble control and annexin 2 siRNA-transfected cells. Annexin 2 knockdown decreased cell spreading by ~45%, and expression of myc-RhoAV14 restored the number of spreading cells to that of controls (**B**, $*P < 0.05$). Expression of myc-RhoAV14 also restored the percentage of wound closure after 16 hours in annexin 2 siRNA-transfected cells (**C** and **D**, $*P < 0.05$). TX-100-soluble and -insoluble pools of actin were analyzed by Western blotting in siRNA-transfected cells with or without myc-RhoAV14 expression. Representative Western blots (**E**) and densitometric analysis of blots (**F**) revealed significant increase in TX-100-soluble actin (G-actin) in annexin 2 siRNA-transfected cells, which was rescued by expression of myc-RhoAV14. No changes in total actin were observed in these groups.

wound closure. For these studies, we used an adenoviral vector encoding N-terminal myc-tagged RhoAV14 and EGFP as well as a stable and inducible Caco-2 cell line expressing EGFP-tagged, constitutively active RhoA (RhoAV14-EGFP). Four days after siRNA transfection and 3 days after adenoviral infection, cells were subjected to spreading assays as above. As shown in Figure 8, A and

B, expression of myc-RhoAV14 + EGFP did not significantly influence the ability of control-transfected cells to adhere and spread on collagen I 2 hours after plating. Annexin 2 siRNA transfection alone resulted in a ~45% reduction in cell spreading, similar to that observed in prior experiments. The expression of myc-RhoAV14 + EGFP in this group restored the number of spreading

cells to that of controls. Similar results were obtained using our stable inducible cell line expressing RhoAV14-EGFP (data not shown). We also examined whether myc-RhoAV14 could prevent the inhibition of wound closure induced by annexin 2 knockdown. Following siRNA transfection, cells grown in 24-well culture plates were infected with adenovirus encoding myc-RhoAV14 + EGFP. Three days after adenoviral transfection, cells were subject to restitution assays as above. As shown in Figure 8, C and D, expression of myc-RhoAV14 restored the percentage of wound closure to that of controls. Expression myc-RhoAV14 in scramble control siRNA-transfected cells did not significantly affect wound closure. Thus, expression of myc-RhoAV14 prevents the inhibition of cell spreading and wound closure induced by annexin 2 knockdown.

We next examined whether expression of constitutively active RhoA could prevent the changes in actin polymerization due to annexin 2 knockdown. Four days following transfection with the respective siRNAs, and 3 days after adenoviral infection, extraction of monomeric actin (G-actin) was performed using 1% TX-100 in HBSS+. As above, these experiments were performed in both subconfluent/spreading Caco-2 monolayers and those 6 hours after wounding with similar results. As shown in Figure 8, E and F, knockdown of annexin 2 resulted in an increase in monomeric actin with no changes in total actin. Expression of myc-RhoAV14 in annexin 2 siRNA-transfected cells restored the G/F actin ratio similar to that of scramble control-transfected cells. Expression myc-RhoAV14 in control cells induced an increase in F-actin content consistent with its known effect of inducing polymerization of basal actin filaments or stress fibers.

Annexin 2 Colocalizes with Rho Along the Submembranous Portion of the Plasma Membrane and Co-Immunoprecipitates with Endogenous and Constitutively Active RhoA

To further explore the relationship between annexin 2 and Rho, we performed localization studies in Caco-2 cells. Rho localized to the cytoplasm of lamellipodial extrusions of migrating cells where it colocalized with annexin 2 (Figure 9A, a–c, arrows). In stationary cells, a distinct pool of Rho colocalized with annexin 2 along the lateral membrane domains (Figure 9A, d–f, arrows). Rho was also seen in the cytoplasm and in the perinuclear region of the cells. This pattern of Rho localization is similar to that reported for other polarized epithelial cell types.³⁷

To evaluate the possibility that annexin 2 interacts with Rho-containing complexes, we performed co-immunoprecipitation experiments. Whole cell lysates of spreading cells were generated using lysis buffer containing 1% Triton X-100 and 0.5% Igelal. Insoluble material was removed by high-speed centrifugation ($14,000 \times g$ for 5 minutes). Endogenous Rho was immunoprecipitated using rabbit polyclonal anti-RhoA antibodies. Immunoblot analysis was performed sequentially on the same membrane, first for Rho to verify successful immunoprecipita-

tion. Immunoblot analysis for annexin 2 was then performed. As shown in Figure 9B, Western blot analysis of endogenous Rho immunoprecipitates demonstrated the presence of annexin 2. Immunoprecipitation using control IgG showed no Rho or annexin 2 protein as expected. These studies were also performed using cell lysates from confluent and wounded monolayers. We did not observe any significant difference in the amount of annexin 2 that co-immunoprecipitated with Rho (data not shown). Immunoprecipitation studies using anti-annexin 2 antibodies were inconclusive due to the limited efficiency of pulldown (~10% of total annexin 2; data not shown). To determine whether annexin 2 co-immunoprecipitates with active RhoA, we induced our Caco-2 cell line to express RhoAV14-EGFP and performed immunoprecipitation experiments using anti-GFP antibodies. As a control, GFP immunoprecipitations in cells expressing EGFP alone were performed. As above, immunoblotting was performed sequentially, first with anti-EGFP antibodies followed by anti-annexin 2 antibodies. As shown in the Western blot analysis in Figure 9C, annexin 2 was identified in the immunoprecipitate of RhoAV14-EGFP and not in EGFP alone. The efficiency of pulldown for both Rho and RhoAV14-EGFP was approximately 80% as determined by densitometric analysis of Western blots of pre- and postimmunoprecipitation lysates. Up to 5% of the total annexin 2 in the whole cell lysates co-immunoprecipitated with endogenous Rho and up to 20% with RhoAV14-EGFP (data not shown). Thus annexin 2 co-immunoprecipitates with endogenous Rho and constitutively active RhoA. Although co-immunoprecipitation studies were performed in cells expressing myc-RhoAV14 using mouse anti-myc antibodies, results were inconclusive due to technical limitations of the available reagents (data not shown).

Discussion

Annexin 2 is a multifunctional protein shown to be involved in diverse cellular processes such as endocytosis/exocytosis, linkage of membrane-associated protein complexes to the actin cytoskeleton, ion channel formation, plasminogen activation, and cell-matrix interactions.^{9,15,26,38–44} Annexin 2 has also been implicated in the migration of various cell types including epithelial cells. Studies in Moloney sarcoma virus-transformed MDCK cells suggest that annexin 2 suppresses cell motility through interactions with Na,K-ATPase and Rac1.¹³ Lewis lung carcinoma cell lines treated with exogenous annexin 2 resulted in an inhibition of cell migration, whereas treatment with annexin 2 antibodies enhanced migration.¹⁴ Other studies suggest that binding of annexin 2 to the extracellular matrix protein tenascin-C promotes cell migration.^{15,16} In the present study, we observed an inhibition of epithelial cell spreading and wound closure following siRNA-mediated down-regulation of annexin 2 expression. These effects were not related to altered β -1 integrin expression, because total levels were not changed on annexin 2 knockdown (data not shown). In addition, we did not observe significant

Figure 9. Annexin 2 colocalizes and co-immunoprecipitates with endogenous Rho and constitutively active RhoA. Wounded and nonwounded Caco-2 monolayers were double-labeled for annexin 2 (red) and Rho (green). In migrating cells (**A, a–c**), annexin 2 colocalized with Rho within lamellipodial extrusion (**arrows**). Scale bar = 20 μ m. In stationary cells (**A, d–f**), a pool of Rho colocalized with annexin 2 along the lateral subcortical region of the cells (**arrows**). Scale bar = 10 μ m. Rho was immunoprecipitated in spreading cells and Western blotted with antibodies to annexin 2 and Rho (**B**). Immunoprecipitation with IgG-matched antibody served as a control. Immunoprecipitation for RhoAV14-EGFP was performed using antibodies to the EGFP tag and Western blotted for Rho and annexin 2 (**C**). Immunoprecipitation of EGFP alone served as a control.

changes in the localization of β -1 integrin in migrating cells following annexin 2 knockdown (data not shown). The functional effects of annexin 2 knockdown were, however, associated with significant alterations in F-actin organization. A major consequence of annexin 2 knockdown was a dramatic decrease in F-actin bundles within lamellipodia and along the base of cells immediately removed from the migrating front. Biochemically, these results were supported by an increase in monomeric actin with no changes in total cellular actin. Given that annexin 2 interacts with various actin cytoskeletal-associated proteins and F-actin itself, knockdown of annexin 2 expression could influence F-actin organization by a number of different mechanisms.^{7,11}

There is evidence to suggest that annexin 2 plays a structural role in determining organization of F-actin. An-

nexin 2 exists as two forms within cells, a monomeric form and a heterotetrameric form consisting of two annexin 2 molecules linked to each other at their N terminus through two p11 light chain subunits.^{45,46} The heterotetrameric form of annexin 2 has been shown to bundle actin filaments *in vitro* and interact with the actin-binding protein spectrin.^{7,10,47,48} Introduction of recombinant annexin 2 into permeabilized pulmonary epithelial cells induces alterations in actin cytoskeletal structure.⁴⁹ Furthermore, siRNA-mediated knockdown of annexin 2 expression in MDCK cells prevents the localization of AHNK, believed to regulate F-actin organization, to the subcortical region and results in structural changes that lead to decreased cell height.³⁴ Our annexin 2 localization studies revealed that a significant pool of annexin 2 is localized in the subcortical F-actin networks and knockdown of annexin 2

expression resulted in alterations of columnar cell morphology. It is therefore possible that annexin 2 is itself an important structural component of F-actin networks in intestinal epithelial cells, or alternatively, could mediate the recruitment/association of structural proteins to the actin cytoskeleton as in the above MDCK cell model.³⁴ Thus, inhibition of annexin 2 expression could result in destabilization of actin filament networks and their subsequent depolymerization. Although annexin 2 is abundantly expressed in lamellipodia and along the base of migrating cells, annexin 2 did not appear to localize to basal F-actin bundles despite its influence on their formation. This suggests that annexin 2 regulates such F-actin networks through other mechanisms. Indeed, annexin 2 has also been shown to interact with and mediate the recruitment of signaling molecules to membranes that in turn regulate the actin cytoskeleton.

Classical signaling molecules that have been shown to regulate the formation of distinct F-actin networks include the GTPases Rho, Rac, and Cdc42. Rac1 and Cdc42 are required for the development of lamellipodia and filopodia, respectively. Rho regulates stress fiber formation, actin-myosin contractile events, and cell-matrix adhesion.^{35,36} Our results suggest that annexin 2 is involved in the membrane association of Rho proteins. Although the induction of Caco-2 cell migration was not associated with significant changes in the levels of membrane-associated Rho (data not shown), this finding could be reflective of redistribution of Rho from one membrane compartment to another, such as from intracellular membranes to the plasma membrane, and does not exclude an important role for membrane-associated Rho in Caco-2 cell migration. Annexin 2 colocalized with Rho within lamellipodial extrusions and along the submembranous region of cells, and following siRNA-induced down-regulation of annexin 2 expression, there was diminished Rho content in membrane preparations. It is therefore possible that annexin 2 could participate in the recruitment or stabilization of Rho to cellular membranes. This idea is supported by previous studies which suggest that annexin 2 mediates the recruitment of signaling molecules such as Rac1 and SHP-2 tyrosine phosphatases to specific membrane domains in MDCK and endothelial cells, respectively.^{12,50} Annexin 2 co-immunoprecipitated with endogenous Rho and constitutively active RhoA, raising the possibility that annexin 2 interacts with Rho-containing protein complexes, particularly RhoA. However, the efficiencies of immunoprecipitation for endogenous Rho and constitutively active RhoA were approximately 80%, and only a small percentage of the total annexin 2 co-immunoprecipitated. This finding could be reflective of a highly dynamic and transient interaction. An alternative explanation is that annexin 2 plays a role in the organization of specific membrane domains required for Rho association and is not directly associated with Rho-containing protein complexes. This notion is supported by studies in smooth-muscle cells in which enzymatic cleavage of annexin 1 and 2 resulted in the release of Rho from membrane compartments.⁵¹ In addition, annexin 2 has been shown to induce clustering of specific plasma membrane

phospholipids and play a role in lipid domain formation.^{52,53}

It is currently thought that the membrane association and activation status of Rho GTPases are linked. Following release from its GDP dissociation inhibitor, Rho can be targeted to membranes where it is subsequently activated.^{54,55} Consistent with this notion, we observed not only a decrease in the amount of membrane-associated Rho but also reduced active Rho following annexin 2 knockdown. This finding supports a role of annexin 2 in mediating Rho membrane association that is required for its activation at these sites. Thus, absence of annexin 2 would therefore influence RhoA-mediated F-actin reorganization, which in turn affects motility of annexin 2 deficient cells. This idea is supported by our findings that expression of a constitutively active RhoA mutant reverses the defect in cell spreading and wound closure induced by annexin 2 knockdown. Interestingly, one of the Rho mutants used for these studies contains a C-terminal EGFP tag that prevents normal prenylation of RhoA. This apparently contradicts the common belief that C-terminal prenylation is required for the appropriate function of Rho proteins.⁵⁶ However, studies using mouse embryonic fibroblasts suggest that prenylation is not required for the proper localization and activation of Rho.⁵⁷ Together, these findings support that other mechanisms independent of C-terminal prenylation regulate targeting/activation of Rho GTPases. In contrast to Rho, we did not identify significant effects on the membrane association/activation status of Rac1 following annexin 2 knockdown.

In summary, we have shown that annexin 2 is up-regulated in migrating intestinal epithelial cells and that functional knockdown of annexin 2 using siRNA inhibits spreading and wound closure of Caco-2 cells. This is associated with alterations in F-actin networks and decreased membrane-associated and active Rho. Rho and annexin 2 colocalize in migrating cells, and annexin 2 co-immunoprecipitated with endogenous Rho and active RhoA. These findings implicate annexin 2 in the regulation of Rho membrane associations that impact Rho-dependent signaling pathways and related actin cytoskeletal remodeling during intestinal epithelial cell migration.

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