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Annexin A2 Regulates β1 Integrin Internalization and Intestinal Epithelial Cell Migration*5

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BACKGROUND: Intestinal epithelial wound closure requires dynamic regulation/turnover of cell-matrix adhesions.

RESULTS: Down-regulation of annexin A2 increases cell-matrix adhesion and delays β1 integrin internalization from the plasma membrane.

CONCLUSION: Annexin A2 promotes wound closure by regulating internalization of β1 integrin in migrating epithelial cells.

SIGNIFICANCE: This study identifies a novel role of annexin A2 in regulating intestinal epithelial cell migration.

The gastrointestinal epithelium functions as an important barrier that separates luminal contents from the underlying tissue compartment and is vital in maintaining mucosal homeostasis. Mucosal wounds in inflammatory disorders compromise the critical epithelial barrier. In response to injury, intestinal epithelial cells (IECs) rapidly migrate to reseal wounds. We have previously observed that a membrane-associated, actin binding protein, annexin A2 (AnxA2), is up-regulated in migrating IECs and plays an important role in promoting wound closure. To identify the mechanisms by which AnxA2 promotes IEC movement and wound closure, we used a loss of function approach. AnxA2-specific shRNA was utilized to generate IECs with stable down-regulation of AnxA2. Loss of AnxA2 inhibited IEC migration while promoting enhanced cell-matrix adhesion. These functional effects were associated with increased levels of β1 integrin protein, which is reported to play an important role in mediating the cell-matrix adhesive properties of epithelial cells. Because cell migration requires dynamic turnover of integrin-based adhesions, we tested whether AnxA2 modulates internalization of cell surface β1 integrin required for forward cell movement. Indeed, pulse-chase biotinylation experiments in IECs lacking AnxA2 demonstrated a significant increase in cell surface β1 integrin that was accompanied by decreased β1 integrin internalization and degradation. These findings support an important role of AnxA2 in controlling dynamics of β1 integrin at the cell surface that in turn is required for the active turnover of cell-matrix associations, cell migration, and wound closure.

The intestinal epithelium functions as a selectively permeable barrier that allows for the absorption of nutrients and fluids while excluding bacteria and toxins from underlying tissue compartments. The epithelium is highly dynamic as it is actively turned over by proliferation of crypt epithelial cells, migration of cells along the crypt-luminal axis, and apoptosis at the luminal surface. Epithelial injury such as that observed in inflammatory diseases leads to fluid and electrolyte loss as well as exposure to luminal antigens that further exacerbate the inflammatory response. It is therefore critical that epithelial wounds efficiently reseal so as to reestablish the intestinal epithelial barrier and restore the physiologic state. The intestinal epithelium has a remarkable capacity to efficiently reseal wounds. An important mechanism of intestinal epithelial wound closure involves migration of the epithelial cell sheet that rapidly covers denuded surfaces (1–3).

Cell migration requires regulated cycles of polarized cell-matrix attachment at the leading edge of the cell with corresponding detachment at its trailing edge. These critical migratory events are mediated by dynamic turnover of integrin-mediated adhesions. Indeed, disassembly of integrin-based cell-matrix adhesions is important for mediating forward cell movement. Past studies have shown that integrin based adhesions form at the leading edge, mature, and then disassemble at the trailing edge. Focal adhesion disassembly requires endocytosis of integrins that are then recycled back to the plasma membrane at the leading edge of migrating cells or destroyed by lysosomal degradation (4–7). A central component of such biological events is the coordinated intracellular movement of proteins allowing for reuse of non-damaged proteins. Studies from others and our group have demonstrated that β1 integrin plays an important role in IEC cell-matrix adhesion and migration (8–10). Additionally, we and others have shown endocytosis and trafficking of β1 integrin is lipid raft-mediated (11, 12).

We have previously reported that the calcium-dependent phospholipid binding protein, annexin A2 (AnxA2), facilitates IEC migration (13). AnxA2 is a highly conserved protein in

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2The abbreviations used are: IEC, intestinal epithelial cell; AnxA2, annexin A2; BafA1, bafilomycin A1; pPaxY118, phosphorylated paxillin at tyrosine 118; eGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; shCtrl, control shRNA; shAnxA2, annexin A2 shRNA.
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invertebrates and plants and is expressed in most cell types. AnxA2 contains four 70–80-amino acid annexin core repeats that allow for Ca²⁺-dependent attachment to phosphatidylinositol, 4,5-bisphosphate-rich membranes, a component of lipid rafts (14–20). Additionally, AnxA2 can cap filamentous actin while associated with lipid rafts (21–23). Because AnxA2 regulates membrane-membrane and membrane-cytoskeletal interactions, it has been implicated in functioning as a regulatory protein for a wide range of membrane trafficking events that include endocytosis, endosome fusion, and exocytosis (24–29).

Although the structure function relationship between AnxA2 and migration has been investigated, the mechanisms by which AnxA2 controls IEC migration remain poorly understood. Using an AnxA2 shRNA gene silencing approach, we identify a role for AnxA2 in regulating the internalization of cell surface β1 integrin that is needed for the turnover of focal adhesions, forward cell movement, and ultimately, wound closure.

EXPERIMENTAL PROCEDURES

Cell Culture and Constructs—Lentiviruses were generated in HEK293T cells by polyethyleneimine transfection (Open Biosystems). Titered lentiviruses were then used to transduce SK-CO15 and Caco2 human colon cancer epithelial cells. Clonal lines were grown as described previously except for the addition of 2 µg/ml puromycin (30). Cycloheximide (MP Biomedical) was used at the concentration of 50 µg/ml for the given amount of time at 37 °C before harvesting cells. Bafilomycin A1 (Acros) was used at the concentration of 30 nM for two hours at 37 °C before fixation. For transient rescue experiments, a pShuttle eGFP plasmid containing a shRNA-resistant AnxA2 (Addgene) was electroporated into cells in solution L, program 15 (Lonza). For scratch wound rescue experiments a shRNA-resistant AnxA2-eGFP was subcloned from pShuttle into pLEX to generate a shRNA-resistant AnxA2-eGFP lentivirus. Cells were transduced with the lentivirus and sorted for eGFP expression by flow cytometry. Supplemental Table 1 contains the shRNA sequences and primers used for the creation of shRNA-resistant constructs.

Immunoblotting—Cells were lysed in radioimmune precipitation assay buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) supplemented with protease and phosphatase inhibitor mixtures (Sigma). Lysates were cleared by centrifugation before boiling in a reducing SDS sample buffer. After SDS-PAGE, resulting gels were transferred to Immobilon-FL PVDF membranes (Millipore) overnight. Immunoblots were incubated in casein for 1 h to inhibit nonspecific binding followed by primary and secondary antibody treatment and scanned (LI-COR Biosciences). The following primary antibodies were used: β1 integrin clone EP1041Y (Novus), AnxA2 clone ZO14 (Invitrogen), paxillin clone 165 (BD), pPaxillin Y118 (2541, Cell Signaling), actin (A2668, Sigma), cyclin D1 clone DCS-6 (Santa Cruz Biotechnology), E-cadherin clone HEC1-1 (in-house), Caveolin-1 clone N-20 (Santa Cruz Biotechnology) and transferrin receptor clone C-20 (Santa Cruz Biotechnology) were used. Secondary antibodies were from Kirkegaard & Perry Laboratories, Inc.

Immunofluorescence Microscopy—Cells were fixed in 3.7% formalin for 20 min and permeabilized in 0.5% Triton X-100 for 3 min. Fixed cells were then incubated with 5% bovine serum albumin for 1 h before primary antibody incubation overnight. After secondary antibody incubation, cells were mounted in ProLong® gold antifade agent (Invitrogen) before confocal microscopy (Zeiss LSM 510). The following primary antibodies were used: pPaxillin Y118 (Cell Signaling), β1 integrin clone Mab13 (BD Biosciences), AnxA2 clone 3E8-B6 (Sigma). Alexa Fluor-conjugated secondary antibodies were purchased from Invitrogen. Proliferation was monitored by a 1-h pulse with 5-ethynyl-2'-deoxyuridine (Invitrogen). Cells were fixed and permeabilized as described above before counterstaining with To-Pro® 3-iodide (Invitrogen) to highlight nuclei. For Rab5a co-localization experiments, cells were transduced in suspension with CellLight® early endosomes RFP, Bacmam (version 2.0, Invitrogen) before culturing. Focal adhesions in individual cells were quantified using To-Pro® 3-iodide to highlight nuclei and NIH ImageJ software.

Scratch Wound Assay—Scratch wounds were generated using suction via a micropipette. Time-lapse imaging of wound closure was then performed. Images of the leading edge were captured every 2 min for 4 h at 37 °C with an Electron Multiplying Charge-Coupled Device camera (Photometrics). For the 24-h time point, the bottom of the culture plate was scored by a marker perpendicular to the wound, and images were taken at this intersection immediately after wounding and 24 h later. NIH ImageJ was used to quantify wound areas.

Extracellular Matrix Adhesion Assay—Single cell suspensions were generated using a non-enzymatic cell dissociation buffer (Invitrogen). Equal concentrations of cells (10000 cells in 0.1 ml) were resuspended in optiMEM with 0.1% BSA and then incubated with BCECF-AM (Invitrogen) according to the manufacturer’s protocol. Antibodies against β1 integrin, clone Mab13 (Becton Dickinson Biosciences) or clone TS2/16 (eBioscience), were dia lyzed in PBS overnight and used at a concentration of 5 µg/ml. Cells were incubated with these antibodies for 2 h prior to the adhesion assay. Antibody-treated cells were then allowed to adhere to a matrix-coated/BSA-blocked 96-well plate for 30 min at 37 °C. Plates were coated overnight with 10 µg/ml of Matrigel™ (356234, BD Biosciences) or fibronectin (354008, BD Biosciences).

Real Time PCR—RNA was isolated using Trizol® (Invitrogen) and treated with DNase I (Qiagen) prior to cDNA synthesis using an oligo(dT) primer and Superscript® III (Invitrogen). SYBR® Green master mix Applied Biosystems was used to perform real time PCR on a Bio-Rad iQ5 cycler. Cycle threshold values (CT) were used to calculate the fold change in mRNA according to the 2⁻ΔΔCT method (31). GAPDH was used as a reference gene. Supplemental Table 1 includes PCR primer sequences.

Lipid Raft Isolation—Cells were harvested in HBSS + supplemented with a protease inhibitor mixture (Sigma) before nitrogen cavitation at 200 psi for 15 min (32). The postnuclear supernatant was placed in the bottom of a 5–35% continuous sucrose gradient. Gradients were then centrifuged at 39,000 rpm for 19 h in a SW41 rotor (Beckman) to isolated light density lipid
rafts (~21% sucrose). Sucrose fractions were harvested and immunoblotted.

Cell Surface Biotinylation and Endocytosis—Pulse-chase biotinylation experiments were performed as described previously (33, 34). Briefly cell surface proteins were biotinylated (0.5 mg/ml) on ice using NHS-SS-biotin (Pierce). Internalization of cell surface proteins was induced by placing the cells at 37 °C for 2 h. Biotin was stripped from non-internalized proteins by treating cells twice with 20 mM 2-mercaptoethane sulfonate sodium (Genscript) for 5 min. Cells were then treated with 20 mM iodoacetamide (Sigma) for 15 min to quench any remaining 2-mercaptoethane sulfonate sodium. Biotinylated proteins from soluble lysates (200 μg) were purified using monomeric avidin agarose (Pierce), eluted in a reducing SDS sample buffer, and subjected to immunoblot analysis.

RESULTS

AnxA2 Promotes Epithelial Cell Migration and Wound Closure—We have previously reported that AnxA2 is up-regulated in migrating IECs and controls cell motility and wound closure (13). To further identify the mechanisms by which AnxA2 regulates epithelial cell motility, we generated cell lines with stable down-regulation of AnxA2 using two different shRNA sequences (shAnxA2) and a control cell line with a non-silencing shRNA target (shCtrl). Additionally, to verify our results, AnxA2 was stably down-regulated in two model IECs (SK-CO15, Caco2). Immunoblot analysis revealed a significant down-regulation of AnxA2 (~80% reduction) in shAnxA2 cells as compared with shCtrl cells (Fig. 1A). Furthermore, to determine whether any effects of the AnxA2 shRNA were specific to loss of AnxA2 protein, we transduced shAnxA2 cells with a shRNA-resistant eGFP-tagged AnxA2. As shown in Fig. 1A, the AnxA2 eGFP fusion protein was identified at 70 kDa using an AnxA2 antibody. Actin and tubulin were used as loading controls. We next tested the functional effects of stable AnxA2 down-regulation and rescue on IEC wound closure. Using an in vitro scratch wound resealing assay and time-lapse imaging, we observed that loss of AnxA2 resulted in a 4-fold delay in wound closure over a four hour time period and re-expression of AnxA2 was able to significantly rescue the delay in wound closure (p < 0.001) (Fig. 1B, supplemental Movies 1–3). Similarly, after 24 h of wound closure, shAnxA2 cells displayed a 2-fold decrease in wound closure that was abrogated in shAnxA2 cells

FIGURE 1. AnxA2 down-regulation inhibits epithelial cell migration and wound closure. A, SK-CO15 epithelial cells lines expressing control non-silencing shRNA (shCtrl) and annexin A2 shRNA (shAnxA2) were generated by lentivirus transduction of the respective shRNA. To re-express AnxA2 in shAnxA2 cells, an shRNA-resistant eGFP fusion with AnxA2 was transduced into shAnxA2 cells. Lysates from representative clones and eGFP expressing cells were immunoblotted for AnxA2 and tubulin. The numbers below the lanes represent fluorescence intensity quantifications. B, scratch wounds were made in epithelial monolayers, and images were taken at the same position every 4 min for 4 h. Data represent mean ± S.E. **, p < 0.005 and ***, p < 0.0001, shCtrl versus shAnxA2. ##, p < 0.001, shAnxA2 versus shAnxA2-AnxA2eGFP. Scale bar, 250 μm.
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re-expressing AnnexA2 (supplemental Fig. 1A). These experiments support a role of AnnexA2 in controlling intestinal epithelial wound closure.

Because wound closure can be mediated by cell proliferation in addition to cell migration, epithelial proliferation was evaluated by determining EdU incorporation in cells during wound closure. Despite decreased wound closure in shAnnA2 cells as compared with control cells, we observed increased proliferation in cells with down-regulated AnnexA2 (supplemental Fig. 1B). These findings suggest that the decreased wound closure following AnnexA2 down-regulation is mediated by an influence of this protein on epithelial cell migration.

Down-regulation of AnnexA2 Increases Cell Matrix Adhesion—Because AnnexA2 facilitates IEC migration that, in turn, requires dynamic turnover of cell–matrix associations, we next investigated the adhesion of epithelial cells to a complex extracellular matrix. Down-regulation of AnnexA2 increased cell–matrix adhesion (40% increase) (Fig. 2A, left panel). In an analogous manner, increased adhesion of shAnnA2 cells to fibronectin was also observed (Fig. 2A, right panel). Because SK-CO15 cells were used for these assays, we verified adhesive properties of another model intestinal epithelial cell line, Caco2. Analogous to SK-CO15 cells, increased cell–matrix adhesion was seen in Caco2 cells with down-regulated AnnexA2 (data not shown). To biochemically link functional cell–matrix adhesion to the activity of proteins that mediate cell–matrix adherence, we evaluated the phosphorylation/activation status of the β1 integrin scaffold protein, paxillin. Phosphorylated paxillin at tyrosine 118 (pPaxY118) has been previously shown to increase in the number adhesions in cells with down-regulated AnnexA2, we co-localized β1 integrin with early endosome antigen 1 (data not shown). To ensure the specificity of the AnnexA2 shRNA, we used small interfering RNA (siRNA) to down-regulate AnnexA2 in Caco2 cells. Additionally, β1 integrin stability was significantly increased after 8 h of cycloheximide treatment in shAnnA2 cells as compared with shCtrl cells (p < 0.05).

Given that down-regulation of AnnexA2 resulted in increased stability of β1 integrin protein, we next determined β1 integrin subcellular localization by immunofluorescence labeling and confocal microscopy. In control cells, β1 integrin is visualized in intracellular punctate, “vesicle-like” structures that were prominent at the leading edge of migrating cells in addition to a small pool in the basolateral membrane. However, in migrating shAnnA2 cells, β1 integrin was localized predominantly in the basolateral membrane of migrating cells (Fig. 3D). These findings suggest that loss of AnnexA2 results in increased β1 integrin relative to total β1 integrin in shAnnA2 cells as compared with control cells (Fig. 3E). The AnnexA2 effects observed were specific for β1 integrin as we did not observe a change in total and cell surface levels of another basolateral plasma membrane–associated protein, E-cadherin. The influence of AnnexA2 loss on total and cell surface β1 integrin levels was also confirmed in another model intestinal epithelial cell line, Caco2 (Fig. 3F).

AnnexA2 Promotes β1 Integrin Localization in Endosomes—Cell surface proteins undergo trafficking into early endosomes. Thus, to further characterize β1 integrin trafficking in cells with down-regulated AnnexA2, we co-localized β1 integrin with Rab5a, a marker of early endosomes. To highlight early endosomes, we used CellLight® early endosome RFP, a Bacmam (version 2.0, RFP-Rab5a) construct was expressed in migrating IECs. In shCtrl cells, a large pool of β1 integrin co-localized with early endosomes. However, in shAnnA2 cells, β1 integrin was distributed in the basolateral plasma membrane with minimal localization in RFP-Rab5a containing early endosomes (Fig. 4A). In keeping with the imaging data, subcellular fractionation of migrating epithelial cells by isopycnic sucrose density gradient sedimentation revealed co-sedimentation of AnnexA2 with β1 integrin and an early endosome protein, early endosome antigen 1. Down-regulation of AnnexA2 decreased co-sedimentation of β1 integrin with early endosome antigen 1 (data not shown). To ensure the specificity of the AnnexA2 shRNA, we performed a rescue experiment to determine whether the expression of a shRNA-resistant eGFP-tagged AnnexA2 in shAnnA2 cells would promote the redistribution of β1 integrin to endosomes. Immunofluorescence labeling and confocal microscopy revealed that in cells lacking eGFP-AnnexA2, β1 integrin resided mainly in the basolateral membrane. In cells
**FIGURE 2. Down-regulation of AnxA2 increases cell-matrix adhesion.** A, single suspensions of shCtrl and shAnxA2 cells were labeled with a fluorescent dye prior to adhesion to either Matrigel-coated (left panel) or fibronectin-coated (right panel) plates. The prewash fluorescent signal was compared with the signal of adherent cells after washing the cells with a buffer. Incubation with functionally non-inhibitory (TS2/16) and inhibitory (Mab13) β1 integrin antibodies was used to determine the contribution of β1 integrin to cell-matrix adhesion. Results represent the mean ± S.D. of three independent experiments. **, p < 0.001. shCtrl versus shAnxA2, NT, no treatment. ***, p < 0.0001. shCtrl versus shAnxA2, TS2/16. ###, p < 0.0001. shCtrl versus shAnxA2, Mab13.

B, cell lysates were immunoblotted with antibodies to pPax.Y118, total paxillin (paxillin), AnxA2, and actin. The bar graph shows fluorescence intensity quantifications that are presented as mean ± S.E.

C, cells were incubated with β1 integrin antibodies for 2 h and then subjected to immunoblottting with antibodies to pPax.Y118, total paxillin (paxillin), AnxA2, and tubulin. Cyclin D1 was used as a positive control. D, immunofluorescence labeling and confocal microscopy of pPax.Y118 (white) in shCtrl and shAnxA2 colonies. To quantify the number of adhesions per field, pPax.Y118 puncta were divided by the total number of nuclei (blue). The data is representative of three independent experiments and expressed as mean ± S.D. **, p < 0.001. Scale bar, 20 μm.
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A

shCtrl shAnxA2

β1 int.

140.0

5.1 12.9

37.2

22.6 2.3

42.0

181.2 174.0

Fold change β1 int.

1.0

shCtrl shAnxA2

B

Fold change β1 int. mRNA

1.0

0.5

shCtrl shAnxA2

C

DMSO

Cyclohex.

4 hr

β1 int.

140.0

kD

5.9 15.3 4.8 13.0

37.2

Cyclin D1

39.2

AnxA2

37.2

55.2

312.7 349.8 337.9 326.0

β1 int.

DMSO

Cyclohex.

8 hr

β1 int.

140.0

kD

27.1 52.8 12.9 32.1

37.2

AnxA2

55.2

Tubulin

200.5 202.9 175.1 153.7

Fold change β1 int. (Cyclohex/DMSO)

4 hr

8 hr

shCtrl shAnxA2 shCtrl shAnxA2

D

β1 integrin

2x Magnify

AnxA2

Merge

shCtrl

shAnxA2

E

No Biotin

Biotin

β1int. (Input)

kD

3.3 5.6 1.7 5.6

150.0

β1 int. (Cell surface)

-0.1 -0.1 0.3 3.4

120.0

E-cad. (Input)

26.2 21.9 25.8 22.8

120.0

E-cad. (Cell surface)

0.1 0.1 15.7 23.2

37.2

AnxA2 (Input)

37.2

Actin (Input)

257.1 200.8 148.6 190.8

Fold change cell surf. β1 int. (Normalized to β1 int. Input)

6.0

shCtrl shAnxA2

F

Caco2

shCtrl shAnxA2

β1int. (Input)

kD

1.1 8.2

150.0

β1 int. (Cell surface)

1.3 9.8

37.2

AnxA2

42.0

Actin

7.6 7.9
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FIGURE 3. Cell surface β1 integrin stability is increased following loss of Annexin A2. A, control non-silencing shRNA (shCtrl) or shAnxA2 migrating epithelial cells were isolated and immunoblotted for β1 integrin, AnxA2, and actin. The bar graph shows densitometric quantification with mean ± S.E. B, after purification of total RNA and cDNA synthesis, real time PCR was performed to determined the relative levels of β1 integrin mRNA in shCtrl and shAnxA2 cells. Relative β1 integrin mRNA levels in shCtrl versus shAnxA2 cells was calculated using the 2−ΔΔCt method. GAPDH was included as a reference gene. Results are presented as mean ± S.E. C, shCtrl and shAnxA2 cells were treated with either dimethyl sulfoxide (DMSO; 1:500) or cycloheximide (Cyclohex.) for the stated amount of time, and cell lysates were immunoblotted for β1 integrin (β1 int.), AnxA2, and tubulin. Results are mean ± S.E. *p < 0.05. D, immunofluorescence labeling and confocal microscopy of AnxA2 and β1 integrin in shCtrl and shAnxA2 migrating epithelial cells (24 h). Representative confocal images of leading edge cells adhering to the cell surface are shown. Scale bar, 20 μm. E, cell surface proteins from shCtrl and shAnxA2 SK-CO15 cells were biotinylated on ice, captured with streptavidin, and immunoblotted to detect β1 integrin (β1 int.), E-cadherin (E-Cad.), AnxA2, and actin. A non-biotinylated (No Bio.) sample was included as negative control. F, cell surface proteins from shCtrl and shAnxA2 Caco2 cells were biotinylated on ice, captured with streptavidin, and immunoblotted to detect β1 integrin (β1 int.), AnxA2, and actin.

FIGURE 4. AnxA2 promotes β1 integrin localization in early endosomes. A, shCtrl and shAnxA2 cells were transduced with a Rab5a-RFP Bacmam 2 baculovirus (red) and then wounded. Immunofluorescence labeling of β1 integrin is shown in green. Cells were analyzed by confocal microscopy. Scale bar, 10 μm. B, shAnxA2 cells were transduced with shRNA-resistant eGFP-tagged AnxA2 (green). Epithelial cells were allowed to migrate for 24 h. Confocal images were taken at the leading edge of cells stained for β1 integrin (red). Cells expressing the eGFP-tagged annexin A2 are highlighted with dashed lines. Asterisks show AnxA2-expressing cells with internalized β1 integrin. Scale bar, 20 μm.

re-expressing AnxA2, as marked by GFP expression, β1 integrin redistributed to endosomal-like vesicle structures (Fig. 4B).

β1 Integrin Internalization Is Delayed in IECs with Down-regulated AnxA2—Because loss of AnxA2 increases the cell surface level of β1 integrin, we investigated whether AnxA2 functions to either increase the rate of β1 integrin internalization or decrease the rate of β1 integrin trafficking back to the cell surface. To distinguish between these two possibilities, we first tested the effect of bafilomycin A1 (BafA1) on β1 integrin localization in shCtrl and shAnxA2 cells. BafA1 inhibits the vacuolar-type H+ ATPases, retarding protein trafficking between endosomes needed for recycling (37). In shCtrl cells, BafA1 treatment increased the intracellular accumulation of β1 integrin. However, in BafA1-treated shAnxA2 cells, β1 integrin primarily localized in the plasma membrane, suggesting that loss of AnxA2 delays β1 integrin internalization (Fig. 5A). To determine the rate of β1 integrin internalization in shCtrl and shAnxA2 cells, biotin pulse-chase experiments were performed. After biotin labeling of cell surface proteins at 4 °C, cells were transferred to 37 °C to induce protein internalization. Cells were then placed back at 4 °C, and biotin was chemically removed (stripped) from non-internalized cell surface proteins, leaving only internalized proteins biotinylated. Biotinylated proteins from resultant lysates were pulled down using avidin agarose and immunoblotted for β1 integrin. To ensure complete biotin stripping from non-internalized proteins, one group of cells was left at 4 °C after labeling and then stripped and lysed (labeled strip). Additionally, to account for internalized and degraded biotinylated protein, cells were lysed directly after the internalization period. In shCtrl IECs, all cell surface β1 integrin was internalized after 2 h at 37 °C. In contrast to shCtrl cells, the rate of β1 integrin internalization was significantly decreased in shAnxA2 cells (2-fold) (Fig. 5B). To better define how AnxA2 regulates the internalization of β1 integrin, we determined whether these proteins co-fractionate with the lipid raft associated protein caveolin-1. Lipid rafts from control and AnxA2 down-regulated cells were isolated by flotation in sucrose gradients. Indeed, immunoblot analysis identified AnxA2 and β1 integrin with caveolin-1 in lipid raft-containing sucrose fractions (Fig. 5C). To verify the specificity of this finding, we immunoblotted fractions for the transferrin receptor as it has been previously reported that this receptor does not associate with lipid rafts (32). In conclusion, these results suggest that AnxA2 and β1 integrin associate with IEC caveolin-1-containing lipid rafts that have been previously shown to mediate β1 integrin internalization from the cell surface (11, 12).

DISCUSSION

Directed migration of a cohesive sheet of epithelial cells is needed to achieve mucosal wound closure. In this report, we identify a novel role of AnxA2 in controlling β1 integrin trafficking that is also required for cell-matrix adhesion and migration of the epithelial sheet. In a previous report, AnxA2 has been implicated in mediating cell-matrix adhesion (38). These authors demonstrated that phosphorylation of AnxA2 induces cell detachment and that this effect requires RhoA. In our current study, we show that AnxA2 modulates β1 integrin endocytosis from the cell surface, thereby promoting integrin trafficking and cell migration. In the absence of AnxA2, β1 integrin internalization into early endosomes is inhibited, thereby resulting in increased cell surface β1 integrin that is available...
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A

B

Cell surface (CS) biotin labeling 4°C

4°C MESNA (strip)

Labeled CS proteins Efficiency of biotin removal Remaining biotinylated protein Endocytosed protein

Surface Strip Non-Deg. Internalized

kD

140

150

37

42

β1 int. (Input)

β1 int. (Pull-down)

AnxA2

Actin

%β1 integrin internalized

shCtrl shAnxA2 shCtrl shAnxA2 shCtrl shAnxA2 shCtrl shAnxA2

C

Lipid rafts

kD

140

37

23

100

β1 int.

AnxA2

Cav-1

TfR
Annexin A2 Regulates β1 Integrin Endocytosis

Efficient cell migration requires the internalization and recycling of integrins, however, the general mechanism of β1 integrin endocytosis in IECs remains incompletely understood. In our study, we observed that β1 integrin and AnxA2 co-fractionate with caveolin in lipid raft fractions, suggesting that in IECs, endocytosis of β1 integrin is mediated via lipid rafts. Furthermore, our results suggest that internalization of β1 integrin into early endosomes is facilitated by AnxA2. These findings are highlighted in a hypothetical model in which AnxA2 association with lipid rafts allows for the internalization of β1 integrin, thereby facilitating focal adhesion turnover in migrating cells (Fig. 6A). Following the loss of AnxA2, focal adhesions containing β1 integrin and paxillin accumulate at the plasma membrane resulting in increased cell-matrix adhesion and decreased cell migration (Fig. 6B). Interestingly, we observed that paxillin steady-state levels mimic that observed for β1 integrin. A functionally inhibitory β1 integrin antibody (Mab13) decreased paxillin protein levels. Thus, the association of paxillin with extracellular matrix engaged β1 integrin decreases the rate at which paxillin gets degraded in the cytosol. The phosphorylation of tyrosine 118 in paxillin was also enhanced in shAnxA2 cells. Because this phosphorylation is observed when β1 integrin is engaged with the extracellular matrix (35), this confirms that β1 integrin is fully functional after loss of AnxA2.

AnxA2 has been implicated in regulating intracellular protein trafficking by promoting endosomal fusion and exocytosis. These studies show that AnxA2 enhances the aggregation and fusion of vesicles (26, 41, 42). Interestingly, previous studies in cells with down-regulated AnxA2 have reported that epidermal growth factor and transferrin can be internalized but accumulates at cell matrix adhesion. Because cells require remodeling of β1 integrin adhesions for movement, such a buildup of β1 integrin following loss of AnxA2 retards cell motility. This finding is analogous to a report indicating that down-regulation of sorting nexin 27 results in disruption of multidrug resistance protein 4 receptor internalization, causing increased steady state levels of this receptor (39). In a previous study, we reported that AnxA2 controls intestinal epithelial wound closure by modulating the small GTPase, RhoA (13). Our current findings are complementary to this report because Rho GTPases control F-actin organization that, in turn, modulates dynamics of membrane raft-associated proteins (40). Interestingly, in our previous report, we also observed that transient and incomplete down-regulation of AnxA2 did not significantly alter β1 integrin protein levels. In contrast, our current findings were derived from stable cell lines having greater than 90% down-regulation of AnxA2, which clearly show significantly increased β1 integrin protein levels. These differences can be explained either by limited ability to detect changes under conditions of low transfection efficiency or that there is a threshold for AnxA2 down-regulation, beyond which massive loss of AnxA2 inhibits β1 integrin trafficking.

FIGURE 5. Down-regulation of AnxA2 delays β1 integrin internalization. A, after treating shCtrl and shAnxA2 cells with BafA1, immunofluorescence labeling of β1 integrin (white) and confocal microscopy were performed. Red arrows highlight intracellular accumulation of β1 integrin. Scale bar, 20 μm. B, a pulse-chase biotinylation assay was performed in shCtrl and shAnxA2 cells to determine β1 integrin internalization rates. Cell surface proteins were biotinylated on ice (Cell surface; CS), and protein internalization was allowed to occur at 37 °C for 2 h. Biotin from non-internalized proteins was chemically removed, and internalized biotinylated proteins were pulled down with avidin agarose and subjected to immunoblotting (Internalized). A stripping control (Strip) was performed to ensure the removal of biotin from non-internalized proteins. To control for protein degradation, a non-degraded control (Non-Deg.) was included after the internalization period. Percent internalization was quantified by adding the degraded fraction to the internalized fraction divided by the surface fraction. A non-biotinylated (No Bio) sample was included as negative control. MESNA, Sodium 2-mercaptoethanesulfonate. The graph represents three independent experiments. Results indicate mean ± S.E.; *, p < 0.05. C, shCtrl cells were lysed by nitrogen cavitation, and membrane raft fractions were isolated by floatation in continuous sucrose gradient (5–30%). The fractions were subjected to immunoblot analysis of β1 integrin (β1 int.), AnxA2, caveolin-1 (Cav-1), and transferrin receptor (TfR).

FIGURE 6. Proposed model for annexin A2-mediated endocytosis of β1 integrin. A, in intestinal epithelial cells, AnxA2 associates with lipid rafts (Rafts) that contain β1 integrin (β1). To promote internalization of rafts, the amino terminus of AnxA2 caps plasma membrane-associated polymerizing F-actin (45–47). Internalized β1 integrin can then be reused at the new leading edge of cells, allowing for efficient cell migration. B, in IECs lacking AnxA2, lipid raft (Raft)-associated β1 integrin (β1) builds up at the plasma membrane. Paxillin then becomes phosphorylated and stabilized as a consequence of the increased adhesion. Without being able to detach from the matrix, IECs lacking AnxA2 do not efficiently migrate to close wounds.
late in late endosomes (41, 43). Because we observed inhibition of cell surface β1 integrin endocytosis after down-regulation of AnxA2, it appears that the function of AnxA2 likely depends on its cargo in the vesicular compartments. Furthermore, our loss-of-function study can only analyze the influence of AnxA2 on the earliest event in β1 integrin endocytic recycling, which we determined to be internalization. We therefore do not exclude a role of AnxA2 in controlling β1 integrin trafficking between other endosomal compartments such as β1 integrin recycling back to the plasma membrane in migrating epithelial cells.

Current knowledge of how AnxA2 functions in the endocytosis of lipid raft-associated proteins is very limited. A single study to date suggested that AnxA2 promotes the internalization of a cell surface receptor (44). To build a more comprehensive model on the role of AnxA2 in endocytosis, studies have analyzed key AnxA2 binding partners. These reports have shown that AnxA2 can simultaneously interact with actin and lipid rafts (14–20). The role of actin in endocytosis and the involvement of lipid rafts in endocytosis have been reported previously (45, 46). Actin-nucleating proteins attach to the plasma membrane, thereby promoting polymerization of actin at these sites. Vesicles are attached to filamentous actin (F-actin), which facilitates inward budding (47). Because AnxA2 can cap filamentous actin while being associated with lipid rafts, we propose that AnxA2 provides a link between F-actin and internalization of β1 integrin from cell surface rafts to early endosomes (see model in Fig. 6A). It is likely that PKC-mediated phosphorylation of AnxA2 plays a regulatory role in the endocytosis of lipid rafts and cell-matrix detachment because PKC has been reported to phosphorylate AnxA2 and also regulate the endocytosis of β1 integrin (38, 48–50).

In summary, our findings implicate an important role of AnxA2 in controlling β1 integrin dynamics, epithelial migration, and wound closure.

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REFERENCES

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