Annexin I Regulates SKCO-15 Cell Invasion by Signaling through Formyl Peptide Receptors

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Annexin 1 (AnxA1) is a multifunctional phospholipid-binding protein associated with the development of metastasis in some invasive epithelial malignancies. However, the role of AnxA1 in the invasion/invasion of epithelial cells is not known. In this study, experiments were performed to investigate the role of AnxA1 in the invasion of a model epithelial cell line, SKCO-15, derived from colorectal adenocarcinoma. Small interfering RNA-mediated knockdown of AnxA1 expression resulted in a significant reduction in invasion through Matrigel-coated filters. Localization studies revealed a translocation of AnxA1 to the cell surface upon the induction of cell migration, and functional inhibition of cell surface AnxA1 using antiserum to the cell surface upon the induction of cell migration, and resulted in a significant reduction in invasion through Matrigel-interfering RNA-mediated knockdown of AnxA1 expression SKCO-15, derived from colorectal adenocarcinoma. Small interfering RNA-mediated knockdown of AnxA1 expression revealed a translocation of AnxA1 to the cell surface upon the induction of cell migration, and functional inhibition of cell surface AnxA1 using antiserum (LCO1) significantly reduced cell invasion. Conversely, SKCO-15 cell invasion was increased by ~2-fold in the presence of recombinant full-length AnxA1 and the AnxA1 N-terminal-derived peptide mimetic, Ac2-26. Because extracellular AnxA1 has been shown to regulate leukocyte migratory events through interactions with n-formyl peptide receptors (nFPRs), we examined the expression of FPR-1, FPRL-1, and FPRL-2 in SKCO-15 cells by reverse transcriptase-PCR and identified expression of all three receptors in this cell line. Treatment of SKCO-15 cells with AnxA1, Ac2-26, and the classical nFPR agonist, formylmethionylleucylphenylalanine, induced intracellular calcium release consistent with nFPR activation. Furthermore, the nFPR antagonist, Boc2, abrogated the AnxA1 and Ac2-26-induced intracellular calcium release and increase in SKCO-15 cell invasion. Together, these results support an autocrine/paracrine role for membrane AnxA1 in stimulating SKCO-15 cell migration through nFPR activation. The findings in this study suggest that activation of nFPRs stimulates epithelial cell motility important in the development of metastasis as well as wound healing.

The ability of epithelial cells to migrate is a critical pathophysiologic event in the development of metastasis and wound healing. Epithelial cell migration requires the coordination of three basic cellular processes: actin cytoskeletal reorganization, dynamic cell-matrix adhesion, and matrix remodeling (1, 2).

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The abbreviations used are: AnxA1, annexin 1; siRNA, small interfering RNA; nFPR, n-formyl peptide receptors; Ac, acetyl; fMLP, formylmethionylleucylphenylalanine; BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; RT, reverse transcriptase; FPRL, n-formyl peptide receptor-like.
MATERIALS AND METHODS

Cell Culture—The human intestinal epithelial cell line, SKCO-15, was grown in high glucose (4.5 g/liter) Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 15 mM Hepes (pH 7.4), 2 mM L-glutamine, and 1% non-essential amino acids as previously described (31, 32). Cells were passaged and seeded on collagen-coated permeable supports or tissue culture-treated plates (Costar, Cambridge, MA).

siRNA Transfections—siRNA pools (4 siRNA duplexes) targeting AnxA1, annexin 2 (AnxA2), and lamin A/C were obtained from Dharmacon (Lafayette, CO). Transfections were carried out using Lipofectamine 2000 in Opti-MEM I media (Invitrogen). Lipofectamine 2000 and siRNA (20 μm stock) were diluted separately in Opti-MEM I at a ratio of 1:25 and incubated at room temperature for 5 min. Equal volumes of the siRNA and Lipofectamine 2000 solutions were then mixed and incubated at room temperature for 15 min with gentle agitation. Subconfluent SKCO-15 monolayers were washed and placed in Opti-MEM I media. Transfection solutions were diluted 1:5 into the cultures for a final siRNA concentration of 80 nm. After an overnight incubation, the monolayers were placed back into complete media.

Antibodies and Other Reagents—Monoclonal anti-AnxA1 and anti-AnxA2 antibodies were obtained from BD Biosciences, mouse anti-lamin A/C and rabbit polyclonal anti-annexin 6 from Santa Cruz Biotechnology (Santa Cruz, CA), rat anti-tubulin antibodies from Serotec (Raleigh, NC), rabbit anti-FPR-1, FPRL-1, and FPRL-2 from Gene Tex (San Antonio, TX). Alexa Fluor 488 and 546-conjugated secondary antibodies were obtained from Invitrogen.

The antiserum, LC01, was raised in sheep against the N-terminal domain of AnxA1 as previously described (33). Control sheep serum was obtained from Rockland Immunoenzymicals (Gilbertsville, PA). Inhibitory anti-JAM-A antibody (J10.4) was generated as previously described (34). Recombinant AnxA1 and Ac2-26 (Ac-AMVESEFLQAFWIEENNEQEVYVTQVK) were generated as previously described (35–37). Scramble control peptide (Ac-YES-ctttgcctgtaactccacct-3) was obtained from Sigma. Alexa Fluor 488 and Alexa Fluor 546 conjugated secondary antibodies were obtained from Invitrogen.

Invasion Assays—Invasion assays were performed using commercially available modified Boyden chambers layered with growth factor-reduced Matrigel (BD Biosciences) according to established protocols (38, 39). Cultures of various confluences were used as a source for cells in these assays that had no effect on their invasiveness (data not shown). The chambers were placed in serum-free media containing 0.1% BSA and incubated at 37 °C for 2 h to hydrate and block the membrane. Cells were trypsinized, washed in serum-free media, and counted. An equal number of cells (1 × 10⁶ cells) were loaded into the upper chamber in serum-free media containing 0.1% BSA. Compete media was placed in the lower well. For experiments using peptides and antibodies, media in both the upper and lower chambers were supplemented at the following concentrations: 1:500 dilution for LC01 and control sheep serum, 10 μg/ml for J10.4, 15 μg/ml for recombinant AnxA1, 33 μM for Ac2-26 and scramble control peptide, 1 μM for FMLP, and 100 μM Boc2. After a 24-h incubation at 37 °C (5% CO₂), the upper chamber was cleared of cells using a cotton swab tip. The inserts were then fixed in a solution of 3.7% paraformaldehyde containing 0.1% crystal violet. The number of cells on the undersurface of the membrane (i.e. invaded cells) were counted using bright field microscopy.

SKCO-15 Monolayer Wounding—For biochemical analysis of AnxA1 expression, wounds were made on confluent monolayers grown in collagen-coated 6-well plates using a wound comb as described previously (40). The wounded comb has multiple fine hollow metal teeth evenly spaced (1 mm) with suction attached to the opposite end of the comb to generate reproducible wounds thereby converting the majority of the monolayer into migrating cells. For immunolocalization studies, linear wounds were created in monolayers grown on 0.33-cm² permeable supports using a sterile pipette tip attached to low suction.

Immunofluorescence and Image Analysis—Immunofluorescence studies were performed on cells grown on 0.33-cm² polycarbonate collagen-coated permeable supports. Cells were fixed in 3.7% paraformaldehyde at room temperature for 20 min. All subsequent steps were carried out at room temperature. Permeabilization was carried out using 0.5% Triton X-100 in HBSS++. Cells were washed with HBSS++ and blocked in HBSS++ containing 3% BSA for 1 h. Primary antibody reactions were performed in 3% BSA in HBSS++ for 1 h at a 1:500 dilution for anti-AnxA1 antibodies. Secondary antibodies were diluted 1:1000 in 3% BSA and incubated for 45 min. Monolayers were then washed and mounted on slides in p-phenylenediamine. Confocal microscopy was performed using the Zeiss LSM 510 microscope (Thornwood, NY).

Isolation and Analysis of Cell Surface-bound AnxA1—Surface-bound AnxA1 was isolated by depletion of extracellular calcium as previously described (25, 41). All steps were carried out at 4 °C using cooled solutions to prevent internalization of membrane-associated proteins that occurs upon calcium depletion (42, 43). Monolayers grown in 6-well plates were washed with cooled HBSS++. The monolayers were then washed once with phosphate-buffered saline (without calcium and magnesium) prior to the addition of 500 μl of phosphate-buffered saline containing 1 mM EDTA per well. The monolayers were then incubated with gentle agitation for 45 min. The supernatants were harvested and subject to high speed centrifugation (100,000 × g, 45 min) to remove any cellular debris. Monolayers were then washed with HBSS++/+ and harvested in 500 μl of RIPA buffer. Equivalent volumes of surface wash and cell lysate were subject to Western blot analysis.

RT-PCR Analysis and Immunoblot Analysis—Total RNA was isolated from SKCO-15 cells using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RT-PCR was carried out using the Super Script III One Step RT-PCR system (Invitrogen). The following primer sets were used: FPR-1: forward, 5'-gaaatctcctggaggaccc-3′, and reverse, 5'-ctttgcttgatccattc-3′ (1024-bp amplicon); FPRL-1: forward, 5'-caggaggtcctgcaag-3′, and reverse, 5'-caggaggtc-
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gctgcaag-3' (678-bp amplicon); FPRL-2: forward, 5'-aggattcaggagaatggcc-3', and reverse, 5'-tgtatgggatcagaccacct-3' (672-bp amplicon). Primers selected for these studies were chosen based on FPR-1, FPRL-1, and FPRL-2 sequence alignments and were blasted against the NCBI data base (Mascot; human) to ensure specificity of amplification.

SKCO-15 cell, neutrophil, and monocyte membrane fractions were used for immunoblot analysis. Cells were nitrogen caviitated (200 p.s.i.; 15 min) and subject to low speed centrifugation to remove nuclei (1000 × g for 10 min). Membranes were isolated through high speed centrifugation of post-nuclear supernatants (100,000 × g for 45 min). The membrane pellets were resuspended in 1% n-octylglucoside by sonication on ice (20% duty cycle; 30% output) and subject to SDS-PAGE followed by Western blot analysis using anti-FPR-1, anti-FPRL-1, and anti-FPRL-2 at dilutions of 1:200.

Intracellular Calcium Release Studies—Intracellular calcium release studies were performed according to established protocols (44, 45). SKCO-15 cells were cultured in collagen-coated 96-well plates. Subconfluent monolayers (~50% confluent) were washed with HBSS+/+ and loaded with 5 μM Fluo-4 (Invitrogen) in HBSS+/+ for 20 min at room temperature. The monolayers were washed and placed in HBSS+/+. Using an Axiovert 200M microscope (Zeiss, Thornwood, NY), fluorescence images were obtained at 1-s intervals for up to 250 s after stimulation. Fluorescence intensities of individual cells or small cell clusters (2–3 cells) were determined using LSM 510 software with enhanced physiology features (Zeiss, Thornwood, NY).

RESULTS

siRNA-mediated Knockdown of AnxA1 Inhibits SKCO-15 Cell Invasion—To determine whether AnxA1 plays a role in epithelial cell migration/invasion, we first tested the effects of inhibiting AnxA1 expression by RNA interference on SKCO-15 cell invasion. AnxA1, AnxA2, and lamin A/C siRNA pools were obtained from Dharmacon. Western blot analysis of SKCO-15 cells 4 days post-transfection with AnxA1 siRNA demonstrated specific and significant reductions in AnxA1 protein levels compared with controls (Fig. 1A). Based on densitometric analysis of Western blots, AnxA1 protein levels were reduced by an average of 80% (data not shown) with no effect on AnxA2 or AnxA6 proteins. Lamin A/C and AnxA2 siRNA-transfected cells showed significant reductions in lamin A/C and annexin 2 protein levels, respectively, with no effect on AnxA1 protein levels as expected.

For invasion assays, cells were trypsinized and washed 80 h post siRNA transfection. Cells were then counted, and an equivalent number of cells (1 × 10⁵) were loaded into growth factor-reduced Matrigel-coated invasion chamber inserts (BD Biosciences) in serum-free media containing 0.1% BSA. Complete media was added to the lower chamber and the inserts were incubated for 24 h at 37 °C (5% CO₂). The inserts were then fixed and stained with crystal violet, and the average number of cells invaded per high power field (×100) was determined using bright field microscopy. As shown in the representative bright field images from these assays, a significantly reduced number of invaded cells were observed in the AnxA1 siRNA-transfected group compared with non-treated and control (lamin A/C) transfected cells (Fig. 1B). On average, AnxA1 siRNA-transfected cells demonstrated a 51% reduction in invasion compared with controls (Fig. 1C). AnxA1 knockdown did not significantly effect SKCO-15 cell migration on non-coated filters (data not shown). Thus, inhibition of AnxA1 expression using siRNA inhibits SKCO-15 cell invasion.

Induction of SKCO-15 Cell Migration Induces Translocation of AnxA1 to the Cell Surface—We next determined the expression and localization of AnxA1 in both stationary and migrating cells to gain insight into how the induction of cell migration influences its distribution and provide insight into how AnxA1 might regulate epithelial cell migration. AnxA1 has been shown to localize to the surface of various cell types where it is believed to be important in its biological functions (36, 46, 47). We therefore localized both intracellular and cell surface AnxA1
using permeabilized and non-permeabilized cells. Confluent monolayers were designated as stationary cells, and to generate motile cells, linear wounds were created in confluent monolayers using a sterile pipette tip. Total AnxA1 was labeled in cells fixed with 3.7% paraformaldehyde and permeabilized with 0.5% Triton X-100, whereas surface AnxA1 was labeled in non-permeabilized cells fixed with 3.7% paraformaldehyde. As shown in Fig. 2A, panel a, polarized stationary cells contain AnxA1 within the cytoplasms as well as along the apical and lateral membrane domains (arrows). In migrating cells, AnxA1 is found within the cytoplasm of lamellipodial protrusions and along cell-cell contacts (Fig. 2A, panel b, arrows). In non-permeabilized stationary cells, AnxA1 labeling was identified along the apical surface (Fig. 2A, panel c). Focally, AnxA1 staining was identified along the lateral membrane (Fig. 2A, panel c, arrowheads) suggesting that AnxA1 is also present on the surface, such as plasma membrane domains. This finding could reflect a limited access of the antibody to the intercellular compartment. In non-permeabilized migrating cells, AnxA1 was distributed diffusely over the surface of lamellipodial protrusions (Fig. 2, panel d).

Because AnxA1 was identified on the surface of SKCO-15 cells by immunofluorescence labeling/confocal microscopy, we next sought to determine the relative amounts of surface and intracellular AnxA1 in stationary and migrating cells using a cellular fractionation approach. Confluent monolayers were wounded and cell migration was allowed to proceed by incubation over a 24-h time period. Non-wounded monolayers were designated as stationary cells. Surface AnxA1 was isolated through depletion of extracellular calcium as previously described (25, 41). As shown in Fig. 2B, cell surface AnxA1 levels are increased 2-h post-wounding and remain increased for 24 h. Based on densitometric analysis, cell surface AnxA1 accounts for ~20% of the total AnxA1 in stationary cells and up to 43% of the total in migrating cells (Fig. 2C). Total AnxA1 were also examined in cells migrating over a 24-h time period. Total AnxA1 levels did not significantly change following the induction of cell migration (Fig. 2D). Total AnxA1 levels were also examined in invaded versus non-invaded control cells using assays as describe above. No significant difference in AnxA1 levels were observed between these groups (data not shown). Thus, a pool of AnxA1 is translocated to the cell surface upon the induction of cell migration.

Functional Inhibition of Cell Surface AnxA1 Inhibits SKCO-15 Cell Invasion—The finding that AnxA1 is translocated to the cell surface upon the induction of migration raises the possibility that this pool of AnxA1 may regulate SKCO-15 cell invasion. To determine whether cell surface AnxA1 regulates SKCO-15 cell invasion, we examined the functional effects of inhibitory AnxA1 antiserum (LC01 (33)) on SKCO-15 cell invasion. Cells were trypsinized, washed, and subjected to invasion assays as above in the presence of LC01 or control sheep serum (1:50 dilutions).
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Additionally, to control for immunoglobulin binding to the cell surface, these assays were also performed in the presence of inhibitory anti-JAM-A antibodies (J10.4) (34). After a 24-h incubation at 37 °C (5% CO₂), cells in the upper chamber were removed and the inserts were fixed and stained with 0.1% crystal violet in 3.7% paraformaldehyde. As shown in the bright field images in Fig. 3A, LC01 induced a reduction in the number of invaded cells as compared with the control groups. The average number of cells invaded per high power field (×100) was determined using bright field microscopy that revealed ~45% reduction in SKCO-15 cell invasion in the LC01-treated group (B).

Recombinant AnxA1 and the AnxA1 N-terminal-derived Peptide Mimetic, Ac2-26, Increases SKCO-15 Cell Invasion—Given that LC01 inhibited SKCO-15 cell invasion, we analyzed the effects of recombinant AnxA1 on SKCO-15 cell invasion and determined whether addition of extracellular AnxA1 could restore the invasiveness of AnxA1 siRNA-transfected cells. Non-treated cells and those 80 h post siRNA transfection were trypsinized and subjected to invasion assays in the presence of AnxA1 (15 μg/ml), added to media in both the upper and lower chambers. After a 24-h incubation, the inserts were processed as above. As shown in Fig. 4, A and B, in non-transfected cells, AnxA1 induced a 1.6-fold increase in the number of invaded cells. Furthermore, addition of AnxA1 to the AnxA1 siRNA-transfected group restored the number of invaded cells to that of non-treated and control siRNA-transfected cells (Fig. 4, A and B). We also tested the effects of the AnxA1 N-terminal-derived peptide mimetic, Ac2-26, on SKCO-15 cell invasion (36, 48). Ac2-26 corresponds to amino acids 2–26 of AnxA1 and has been shown to mimic the biologic activity of the full-length protein (36, 49). Incubation of SKCO-15 cells with Ac2-26 does not affect cell surface levels of endogenous AnxA1 (data not shown). Cells were trypsinized and subjected to invasion assays in the presence of Ac2-26 (33 μM), scramble control peptide (33 μM), or no treatment. Peptide was added to both the media in the upper and lower chambers. The inserts were incubated for 24 h prior to processing.

As shown in the representative bright field images in Fig. 4B, middle, the number of cells invaded increased in the presence of Ac2-26 as compared with the non-treated and scramble control treated groups. Ac2-26 induced a 1.7-fold increase in SKCO-15 cell invasion (Fig. 4B, bottom). These findings further support a role for cell surface/extracellular AnxA1 in regulating SKCO-15 cell invasion.

SKCO-15 Cells Express nFPRs—AnxA1 and Ac2-26 have been identified as ligands for nFPRs and regulate leukocyte transendothelial migration through interactions with these receptors (50–52). The mammalian FPR family includes formyl peptide receptor-1 (FPR-1), formyl peptide receptor-like-1 (FPRL-1), and formyl peptide receptor-like-2 (FPRL-2). AnxA1 has been shown to activate FPRL-1, whereas Ac2-26 has been shown to activate all three nFPRs (37, 51). Because extracellular AnxA1 and its peptide mimic increased SKCO-15 cell invasion, we sought to examine whether nFPRs played a role in mediating this effect. We therefore examined the expression of nFPRs in SKCO-15 cells through RT-PCR and Western blot analysis. Total RNA was isolated from SKCO-15 cells and subjected to RT-PCR analysis for FPR-1, FPRL-1, and FPRL-2. As shown in Fig. 5A, amplicons corresponding to the predicted size were amplified from SKCO-15 cells (1024 bp for FPR-1; 678 bp for FPRL-1; 672 bp for FPRL-2). We next performed Western blot analysis of SKCO-15 cell membrane fractions using commercially available rabbit polyclonal antibodies with neutrophil and monocyte membrane fractions as controls. FPR-1 has been reported to run as a broad band in the 50–60-kDa range (53, 54). In neutrophils, FPR-1 has a molecular mass of ~38 kDa that undergoes heterogenous N-terminal glycosylation that increases its mass on SDS-PAGE to different extents (53, 54). In neutrophils, FPR-1 has been reported to run as a broad band in the 50–60-kDa range (55). Western blot analysis of SKCO-15 cell and neutrophil membrane fractions using rabbit poly-
FIGURE 4. **AnxA1 and Ac2-26 increase SKCO-15 cell invasion.** Non-treated and siRNA-transfected SKCO-15 cells were subject to invasion assays in the presence of AnxA1 (15 μg/ml) (A and B). Representative bright field images from these assays show that AnxA1 significantly increased the number of invaded cells in the non-transfected group while restoring the invasiveness of AnxA1 siRNA-transfected cells to that of non-treated and control siRNA-transfected cells (A). The average number of cells invaded was increased by ~1.6-fold in the presence of AnxA1 (15 μg/ml) (B). AnxA1 siRNA-transfected cells exhibited a ~45% reduction in invasion that was rescued by the addition of AnxA1 (15 μg/ml) (B). As shown in the bright field images, Ac2-26 increased the number of invaded cells compared with controls (C). Quantitation of the number of invaded cells using bright field microscopy revealed a 1.7-fold increase in cell invasion in the presence of Ac2-26 compared with scramble control and non-treated cells (D).
clonal FPR-1 demonstrated a single, relatively sharp band at 50 kDa consistent with FPR-1 expression (Fig. 5B). It must be noted that the antibodies used for this analysis were generated against an extracellular epitope using a synthetic peptide as per the manufacturer (GeneTex, San Antonio, TX). Thus, glycosylation could affect the ability of this antibody to bind its epitope and could therefore account for the characteristics of the band that corresponds to FPR-1 identified with this antibody. FPRL-1 has molecular mass of ~39 kDa and we identified a single band in the 37–50-kDa range in both neutrophils and SKCO-15 cell membranes consistent with FPRL-1 (Fig. 5B). For FPRL-2, monocytes were used as a positive control because neutrophils do not express FPRL-2 (56). FPRL-2 has a molecular mass of ~40 kDa. Western blot analysis of SKCO-15 cell, monocyte, and neutrophil membrane preparations using rabbit polyclonal anti-FPRL-2 revealed a prominent band below the 50-kDa marker and another less prominent band above the 50-kDa marker in monocyte and SKCO-15 cell membranes and not in neutrophil membrane preparations (Fig. 5B). These bands are larger than the predicted parental FPRL-2 protein and possibly represent glycosylated species. Taken together, the RT-PCR and Western blot data strongly support expression of FPR-1, FPRL-1, and FPRL-2 proteins in SKCO-15 cells. To determine whether changes in AnxA1 expression influence nFPR protein levels, we analyzed the expression of FPR-1, FPRL-1, or FPRL-2 in siRNA-transfected cells. Four days post-transfection with control (lamin A/C) or AnxA1 siRNA, membrane fractions were subjected to Western blot analysis. No significant changes in FPR-1, FPRL-1, or FPRL-2 expression were observed following AnxA1 knockdown (Fig. 5C).

AnxA1, Ac2-26, and Other nFPR Agonists (fMLP and WKYMVm) Induce Intracellular Calcium Release in SKCO-15 Cells—nFPRs belong to the seven-transmembrane domain Gi-coupled receptor family that when activated, induce intracellular calcium mobilization (57–60). Because we identified expression of nFPRs in SKCO-15 cells, we examined whether AnxA1, Ac2-26, and other known nFPR agonists induce their activation through analysis of intracellular calcium release. For these studies, we utilized the classical nFPR agonist, fMLP, and the peptide agonist WKYMVm (m is D isomer) (61). fMLP stimulates FPR-1 and FPRL-1, whereas Ac2-26 and WKYMVm stimulate FPR-1, FPRL-1, and FPRL-2 (51, 62, 63). AnxA1 has been shown to stimulate FPRL-1 (37). SKCO-15 cells were loaded with Fluo-4 (5 μM, Invitrogen) and fluorescence images were captured once every second for up to 250 s following stimulation. As shown in Fig. 6, A and B, AnxA1 (15 μg/ml) induced a burst of intracellular calcium release, whereas the control group (vehicle only) did not. The nFPR antagonist, Boc2 (100 μM) (64, 65), significantly attenuated the AnxA1-induced intracellular calcium release demonstrating that this effect is mediated through nFPR activation (Fig. 7, A and B). Similar results were obtained using Ac2-26 (Fig. 6, C and D). Interestingly, however, AnxA1 induced a more sustained intracellular calcium release than Ac2-26. This finding may be reflective of a membrane binding component for full-length AnxA1 that subsequently affects its interaction with nFPRs. Both fMLP (1 μM) and WKYMVm (1 μM) also induced intracellular calcium mobilization, whereas cells treated with vehicle only (Me₂SO) showed no response (Fig. 6, E and F). These findings demonstrate that expressed nFPRs in SKCO-15 cells are functional.

nFPR Activation Stimulates SKCO-15 Cell Invasion—To demonstrate that the effects of AnxA1 and Ac2-26 on SKCO-15 cell invasion are mediated through nFPR activation, we examined whether Boc2, a non-selective nFPR antagonist, could block the AnxA1 and Ac2-26-induced increase in SKCO-15 cell invasion. Invasion assays were performed in the presence of AnxA1 (15 μg/ml), scramble control peptide, and Ac2-26 (33 μM) with and without Boc2 (100 μM). As

![FIGURE 5. SKCO-15 cells express FPR-1, FPRL-1, and FPRL-2. RT-PCR analysis of total RNA isolated from SKCO-15 cells revealed the presence of FPR-1, FPRL-1, and FPRL-2 transcripts (A). Western blot analysis of SKCO-15 membrane fractions demonstrates the expression of FPR-1, FPRL-1, and FPRL-2 proteins (B). Western blot analysis of SKCO-15 cell membrane fractions 4 days post siRNA transfection revealed no changes in FPR-1, FPRL-1, and FPRL-2 protein levels due to AnxA1 knockdown (C).](image-url)
shown in Fig. 7, A and B. AnxA1 induced a 1.9-fold increase in SKCO-15 cell invasion compared with controls consistent with previous experiments. Cells treated with Boc2 in addition to AnxA1 exhibited a reduced number of invaded cells approaching that of controls. The presence of Boc2 alone inhibited SKCO-15 cell invasion by an average of 30% compared with the non-treated control (*, p value < 0.05). Thus, Boc2 attenuates the AnxA1-induced increase in SKCO-15 cell invasion consist-
ent with this effect being mediated through nFPR activation. Similar results were obtained using Ac2-26 (Fig. 7, C and D).

We next tested the effects of fMLP on SKCO-15 cell invasion to further support a role for nFPR signaling in regulating this process. SKCO-15 cells were trypsinized and subject to invasion assays (as above) in the presence of 1 μM fMLP. As shown in Fig. 7, E and F, the presence of fMLP increased the number of cells invaded by ~1.6-fold compared with controls. Together, the above data implicate a role for nFPRs in regulating SKCO-15 cell invasion.

DISCUSSION

AnxA1 is a multifunctional protein shown to regulate a variety of cellular processes in epithelial cells including endocytosis/exocytosis, proliferation, differentiation, as well as eicosanoid production (19, 20, 22, 23, 25). AnxA1 expression has also been correlated with the development of metastasis in lung, breast, and head and neck squamous cell cancers (26, 27, 30). Although AnxA1 has been shown to modulate drug resistance in tumors and regulate cell proliferation, its association with the development of metastasis in some malignancies suggests that AnxA1 regulates the migration/invasion process (20, 66). In our study, siRNA-mediated knockdown of AnxA1 expression resulted in a significant inhibition of SKCO-15 epithelial cell invasion demonstrating its role in regulating this process. We subsequently characterized its expression in both stationary and migrating cells to determine how the establishment of cell migration affected its distribution, thereby providing insight into how AnxA1 might regulate epithelial cell migration. AnxA1 has been shown to localize to the cell surface of various cell types including leukocytes, endothelial cells, lung epithelial cells, and synoviocytes where it is thought to be important in its biological function (67–72). We therefore explored cell surface expres-

FIGURE 7. The AnxA1- and Ac2-26-induced increase in SKCO-15 cell invasion is mediated by nFPR activation. SKCO-15 cells were subject to invasion assays in the presence of AnxA1 (15 μg/ml) with or without Boc2 (100 μM) (A and B). Representative bright field photomicrographs (A) show an increased number of cells in the AnxA1-treated group. Quantitation of the number of invaded cells revealed a 1.9-fold increase in invasion in the presence of AnxA1 alone that was significantly attenuated by Boc2 supporting that the AnxA1 increases invasion by nFPR activation (B). Boc2 alone reduced the number of invaded cells by 30% (B), similar results were obtained using Ac2-26 (33 μM) and scramble control peptide (33 μM) (C and D). Invasion assays were performed in the presence of fMLP (1 μM), which increased SKCO-15 cell invasion (E and F).
sion of AnxA1 in SKCO-15 cells. We found that migrating SKCO-15 cells exhibit increased cell surface AnxA1, whereas total AnxA1 levels remain unchanged during cell migration. Thus, AnxA1 is translocated to the cell surface upon the induction of cell migration suggesting a role for extracellular AnxA1 in regulating SKCO-15 cell migration/invasion. Indeed, we found that functionally inhibitory AnxA1 antisera inhibited SKCO-15 cell invasion, whereas the addition of full-length AnxA1 and the AnxA1 peptide mimetic, Ac2-26, significantly increased the invasiveness of SKCO-15 cells. These findings demonstrate that extracellular AnxA1 can regulate SKCO-15 cell invasion. The mechanisms by which AnxA1 is secreted or translocated to the cell surface are currently poorly understood.

The regulatory action of cell surface or extracellular AnxA1 on leukocyte transendothelial migration has been shown to be mediated by signaling through nFPRs (51, 64, 73). nFPRs belong to the seven-transmembrane domain G-protein-coupled receptor family. Three human nFPR family members have been identified and include FPR-1, FPRL-1, and FPRL-2. Although nFPRs are classically thought to act as chemotactic receptors regulating leukocyte migration, they have been shown to be expressed in diverse cellular populations and bind a variety of exogenous and endogenous ligands that elicit differential biological responses (74). AnxA1 and its peptide Ac2-26 are endogenous nFPR ligands, with the shorter fragment reported to activate all three nFPR family members (51). Other host-derived agonists include serum amyloid A, lipoxin A4, amyloid β (Ab), and urokinase-type plasminogen activator receptor, which agonize FPRL-1 (74–78). The classic bacterial derived FPR-1 ligand, fMLP, has also been shown to activate FPRL-1, albeit with significantly reduced affinity (79). Other infectious agent-derived agonists include human immunodeficiency virus, type 1 envelope proteins and the Helicobacter pylori peptide Hp (2–20). WKYMVm is an agonist for all three nFPR family members that were derived from peptide libraries (80, 81). Given this diverse array of nFPR ligands and their expression in various cell types, the role of nFPRs in disease pathophysiology remains to be elucidated.

Expression of nFPRs has been identified in some epithelial cell types including lung epithelia and hepatocytes (82, 83). Immunohistochemical studies using rabbit polyclonal anti-FPR-1 antibodies suggest that it is also expressed in small intestinal epithelial cells (84). Expression of FPRL-1 has also been detected in intestinal epithelial cells, whereas FPRL-2 expression seems restricted to macrophages and dendritic cells (56, 85–87). In this study, we identified expression of all three nFPRs in SKCO-15 cells through RT-PCR analysis and Western blot analysis. Furthermore, the fact that AnxA1, Ac2-26, fMLP, and WKYMVm induced intracellular calcium mobilization demonstrates that these receptors are functional. In lung epithelial cells and hepatocytes, activation of nFPRs by fMLP and AnxA1 induces the production of acute phase reactant proteins (82, 83). In fibroblasts, fMLP stimulation has been shown to increase their adhesion and motility, as well as induce a chemotactic response in a dose-dependent fashion (59). In this study, AnxA1 and Ac2-26 induced intracellular calcium release and increased SKCO-15 cell invasion toward a gradient of serum in a Boc2-sensitive manner. The presence of fMLP also increased SKCO-15 cell invasion. Taken together, these findings demonstrate a role for nFPR signaling in regulating SKCO-15 cell invasion. Thus, in some tumors, AnxA1 and other endogenous nFPR agonists may promote invasion and metastasis through activation of nFPRs in an autocrine/paracrine fashion. Secreted AnxA1 could bind the cell surface and laterally interact with nFPRs via its N terminus, as the N-terminal peptide mimetic activates these receptors. Indeed, during neoplastic progression, numerous autocrine/paracrine signaling pathways are established that promote the ability of tumor cells to invade and eventually colonize new tissues to form metastasis (9, 88). Additionally, following inflammatory insults to mucosal tissues, stimulation of epithelial nFPRs by endogenous ligands such as AnxA1 may promote epithelial cell migration events important in wound healing. For example, it has been shown that inflammation of colonic mucosa, as occurring in inflammatory bowel disease, results in increased AnxA1 production and secretion by infiltrating leukocytes (89, 90). This secreted AnxA1 could stimulate nFPRs expressed by intestinal epithelial cells and promote restitution.

Because nFPRs signaling regulates chemotactic responses in leukocytes and fibroblasts (59, 79), we explored whether Ac2-26 and fMLP stimulate directed migration in SKCO-15 cells. Chemotaxis assays were performed according to established protocols with various modifications to allow adequate time for SKCO-15 cells to adhere and migrate over the filter membranes (59, 91). SKCO-15 cell migration was not stimulated toward gradients of Ac2-26 and fMLP (data not shown). Thus, nFPR activation in SKCO-15 cells induces a chemokinetic response rather than a chemotactic response.

A critical event underlying cell migration is filamentous actin (F-actin) reorganization. In leukocytes, extracellular signals transmitted through nFPRs induce polarized actin filament assembly that requires phosphoinositide 3-kinase and Rho GTPase activity (92, 93). In other cell types, such as A549 lung cancer cells and fibroblasts, stimulation with fMLP and AnxA1 has been shown to induce an increase in F-actin content (59, 83). It is therefore likely that nFPR signaling also regulates the motility of SKCO-15 cells and other epithelial cell types by stimulating actin filament assembly. The signaling cascades by which nFPRs may regulate actin polymerization in epithelial cells is an interesting focus of a future study.

In summary, we have shown that the induction of SKCO-15 cell migration induces a translocation of AnxA1 to the cell surface and inhibitory AnxA1 antisera inhibits SKCO-15 cell invasion. AnxA1, and the AnxA1 peptide mimetic, Ac2-26, stimulates both SKCO-15 cell invasion and intracellular calcium release in a Boc2-sensitive manner consistent with signaling through nFPRs, the expression of which were identified in SKCO-15 cells. These data implicate an autocrine/paracrine role for membrane AnxA1 in stimulating SKCO-15 cell migration by activating nFPRs. These findings also raise the possibility that both endogenous and exogenous nFPR ligands could regulate epithelial cell migration events important in pathophysiologic events such as tumor metastasis and wound healing.
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