Differential Effects of Cytohesin 2 and 3 on β1 Integrin Recycling
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ARF6 regulates the endocytosis and recycling of a variety of proteins, and also promotes peripheral actin rearrangements and cell motility. ARF6 is activated by a large number of GEFs, which likely regulate ARF6 at different locations and during different processes. In this study, we investigate the roles of the cytohesin ARF-GEFs during the recycling of integrin β1. Intriguingly, we find that knockdown and overexpression of ARNO/cytohesin 2 and GRP1/cytohesin 3 have opposing effects on cell adhesion and spreading on fibronectin and on cell migration. We find that ARNO/cytohesin 2 is required for integrin β1 recycling while GRP1/cytohesin 3 is dispensable for this process. This is the first demonstration of unique roles for these proteins.

ARFs (ARF1-6) are small GTPases of the Ras superfamily and regulators of vesicular transport. There are six known ARF isoforms divided into three classes. Class I ARFs (ARFs 1-3) regulate the assembly of vesicle coat complexes in the secretary pathways and at endosomes. Class II ARFs (ARFs 4 and 5) are still poorly understood. The only member of class III, ARF6, is localized at the cell periphery and regulates trafficking between the plasma membrane and endosomal system (1). ARF6 also regulates assembly of the cytoskeleton and cell motility (2-5).

ARF6 regulates the endocytosis of β1 integrin and a number of other membrane proteins. These proteins are internalized via clathrin independent endocytosis. They then join with clathrin dependent cargo in the early endosome where they are sorted either for degradation in the lysosome or for recycling to the plasma membrane (6).

GTPases are molecular on/off switches that cycle between active GTP and inactive GDP bound forms. GTPases require accessory proteins to facilitate activation or inactivation. Activation of GTPases is catalyzed by guanine nucleotide-exchange factors (GEFs), and inactivation requires GTPase activating proteins (GAPs) (7-8). ARF GEFs are referred to as the Sec7 family
based on homology of their catalytic domains to the yeast protein Sec7p. The human genome encodes 15 Sec7 family members (9). Cytohesins are one family of ARF GEFs. The family has 4 isoforms, cytohesin 1, cytohesin 2/ARNO, cytohesin 3/GRP1 and cytohesin 4. The cytohesins are similarly sized (45-50kDa), and extensively conserved (68% identity). Additionally, they share a common domain structure including coiled coil, sec7, PH and basic domains (9-10). Cytohesins are known to regulate actin rearrangements and cell motility (3, 11-12). Cytohesin 1 regulates the spreading and transendothelial migration of lymphocytes, and cytohesin 2/ARNO induces broad lamellapodia and cell migration in Mardin-Darby Canine kidney (MDCK) epithelial cells (5, 13).

In epithelial tissues, cells are attached to each other by cell-cell adhesions and further anchored by cell-substratum adhesions (14-15). These cells become migratory during developmental morphogenesis, wound healing and cancer metastasis and when motile, extensively remodel their adhesions (14). ARF6 regulates adhesion remodeling by controlling the endocytosis and recycling of adhesion proteins (16-17). Adhesion remodeling is one mechanism to facilitate cell migration (18).

Integrins are cell adhesion proteins that interact with matrix proteins such as fibronectin. Cell surface levels of integrins are determined by a balance between endocytosis and recycling. Integrin trafficking may promote cell migration by internalizing integrins at the trailing edge of the cell and recycling them out to the leading edge (19-20). Integrin β1 is a component of the major receptors of fibronectin. ARF6 has been shown to regulate both the internalization and recycling of integrin β1 (17, 21). Recent studies demonstrated that Brag2 activates ARF6 to regulate integrin β1 endocytosis while an unknown GEF activates ARF6 to regulate integrin β1 recycling (22). The established roles for cytohesins and ARF6 in motility suggest that cytohesins could activate ARF6 to regulate integrin β1 recycling during cell migration.

In this study, we test this hypothesis and demonstrate that cytohesin function is required for integrin β1 recycling. Furthermore, we found that cytohesin 2/ARNO but not cytohesin 3/GRP1 is required in integrin β1 recycling. We also show that ARNO and GRP1 have opposing effects on cell adhesion, spreading and migration. ARNO knockdown inhibits cell migration, while GRP1 knockdown increases cell migration. To our knowledge, this is the first demonstration that these two similar proteins, ARNO and GRP1, have unique functions. These findings outline not only the endocytic recycling pathway of integrin β1 but also demonstrate that ARF6 activation under extensive regulatory control
by multiple GEFs during cell migration.

**EXPERIMENTAL PROCEDURES**

*Cells and reagents*- Hela cells were cultured in DMEM supplemented with penicillin, streptomycin, fungizone, 10% FBS, glutamine and NEAA. MCF-7 cells were maintained in DMEM/F12 supplemented with penicillin, streptomycin, fungizone, 10% FBS and NEAA. The T23 line of MDCK cells were maintained in DMEM supplemented with penicillin, streptomycin, fungizone and 10% FBS. All cell lines were cultured at 37°C, 5% CO₂ incubator. SecinH3 was purchased from EMD. The TS2/16 anti-β1 integrin antibody was a gift from Victor Hsu (Brigham and Women’s Hospital). AlexaFluor-647 conjugated TS2/16 was purchased from BioLegand (San Diego, CA).

*siRNA and plasmids*- siRNA duplexes against the sequence GCAAUGGGCCAGGAA-GAAGU targeting human and canine ARNO, and siRNA duplexes against the sequence GGAGAAGGCCUAUAAAAGA targeting human and canine GRP1 were obtained from Dharmacon. Control siRNAs against firefly luciferase or scrambled sequences (siControl 1) were also obtained from Dharmacon. The siRNAs were transfected with Lipofectamine RNAiMax for Hela and MCF-7 cells and with lipofectamine 2000 for T23 MDCK cells using the manufacturer’s suggested protocol for reverse transfection (Invitrogen). Experiments were carried out 48 hours after transfection with siRNAs. Myc-tagged ARNO and flag-tagged GRP1 were obtained from James Casanova. Transient transfections were performed using lipofectamine 2000 or the Neon transfection system (Invitrogen). Experiments were carried out 24 hour after transient transfection.

*Adhesion assay*- Hela cells were transfected with myc-tagged ARNO or flag-tagged GRP1 or with siRNAs targeting ARNO or GRP1. After 24 hours of expression or 48 hours of knockdown, confluent cells were non-enzymatically harvested and dissociated into single cells by treatment with 1mM EGTA/4mM EDTA in PBS for 20 min at 37°C. Harvested cells were then resuspended in serum-free medium. 5x10⁴ cells in 100μl were plated in 96 well plates in wells coated with different concentrations of fibronectin. The cells were allowed to attach for 1 hour and then fixed with 4% formaldehyde. The fixed cells were stained with 0.1% crystal violet. The dye was extracted into 1% sodium deoxycholate and quantitated by measuring the optical density at 590nm.

*Analyzing integrin β1 recycling by microscopy*- Integrin β1 specific antibody TS2/16 was bound to the surface of serum starved cells at 4°C, and then the cells were warmed to make the cells internalize the antibody bound integrin. Residual integrin antibodies remaining on the cell surface were
removed with an acid wash (0.5% acetic acid, 0.5M NaCl pH3.0) on ice, and then 20% FBS was applied to initiate integrin β1 recycling at 37°C (17). Duplicate samples were fixed at 0 and 5min after the initiation of integrin β1 recycling. One sample was permeabalized by 0.1% triton to observe total antibody bound integrin while the other sample was left unpermeabalized to observe surface integrin β1. Both samples were stained with AlexaFluor 546-conjugated goat anti-mouse secondary antibody. Cells were observed and photographed using a Zeiss AxioPlan microscope equipped with a Spot RT3 camera (Diagnostic Instruments). During each experiment standardized microscope and camera settings were used and all images were recorded using identical exposure times. Scale bars were added using ImageJ.

**Biochemical integrin β1 recycling assay**- Antibody binding, integrin internalization and integrin recycling were performed as described above except for fixation of the samples. Instead, recycled integrin antibodies at the surface were removed by a second acid wash after 0 and 5minutes of recycling. Then, the cells were lysed, and the lysate was incubated with protein G-sepharoses to isolate internal antibody bound integrin β1 (17). Precipitated integrin β1 was analyzed by Western blot for integrin β1.

**Surface integrin β1 labeling assay**- Integrin β1 specific antibody TS2/16 was bound to the cell surface at 4°C, and then the cells were washed with serum free media twice. The cells were lysed, and the lysate was incubated with protein G-sepharose to isolate surface antibody bound integrin β1 (17). Precipitated integrin β1 was analyzed by Western blot for integrin β1.

**Migration assay**- Cell migration was assessed using Oris migration chambers (Oris™ Universal Cell Migration Assembly Kit: Platypus Technologies, Madison, WI). T23 cells were transfected with siRNAs targeting ARNO or GRP1 for 2 days. Cells (1x10^5) were then seeded into each test well and incubated at 37°C in a 5% CO2 humidified chamber to permit cell attachment. After 24 hours, all well inserts were removed, and the media was refreshed. The cells were incubated for 24 hours to permit cell migration and then fixed with 4% formaldehyde. The fixed cells were stained with 0.1% crystal violet. Images of each chamber were taken, and cell migration was quantitated by measuring the remaining cell-free surface area using ImageJ (23).

**Spreading assay**- MCF-7 cells were transfected with siRNA targeting ARNO or GRP1. The cells were incubated for two days and then non-enzymatically harvested and dissociated into single cells as described above for the adhesion assay. Harvested cells were then resuspended in serum-free medium and replated on coverslips coated with 10µg/ml of fibronectin. The cells were allowed to spread
for 30 min at 30% confluency and then fixed with 4% formaldehyde. The samples were stained with rhodamine-conjugated palladin and images of each spreading were taken. Cell spreading was quantitated by measuring cell surface area with ImageJ (23).

**Flow Cytometry** - Cell surface integrin levels were measured by flow cytometry. Hela cells were transfected with GFP, GFP + ARNO, or GFP + GRP1. Cells were detached by using 1 mM EGTA/4 mM EDTA in PBS for 10 min on ice. Harvested cells were washed with PBS and fixed in cold 4% paraformaldehyde for 15 min at 4°C while rotating. Cells were stained with AlexaFluor 647-conjugated anti Integrin β1 (TS2/16) antibody for 1 hr at room temperature. After staining the cell suspension was filtered through a 37 μm mesh to remove multi-cell aggregates. The median level of AlexaFluor 647 fluorescence in GFP positive cells was determined by flow cytometry on an FC500 benchtop cytometer.

**RT-PCR** - Total RNA was isolated using the RNeasy kit (Qiagen). Custom primers to amplify bases 156-505 of human GRP1, bases 272-571 of canine GRP1, bases 548-872 of human ARNO, bases 532-872 of canine ARNO and ReadyMade primers to amplify GAPDH were obtained from Integrated DNA Technologies. RT-PCR was performed with 0.5 μg total RNA as template using the Qiagen One-Step RT-PCR kit.

**Deconvolution microscopy** - MCF-7 cells were co-transfected with Flag-GRP1 and myc-ARNO. The cells were spread on fibronectin for 30 minutes as described above for the spreading assay. The cells were then fixed and stained with M2 anti-flag conjugated to FITC and biotinylated-9e10 anti-myc followed by staining with AlexaFluor 546-conjugated streptavidin. The cells were imaged with an Olympus IX 81 microscope and a Hamamatsu Orca camera. Z-stacks with 0.3μm spacing were obtained. Out of focus light was removed using the Constrained Iterative Deconvolution algorithm in Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO). Merged images through the Z and X axis were created using a maximum projection and scale bars were added using Slidebook.

**Results**

**Cytohesins are critical for cell adhesion to fibronectin** - Integrin β1 is a component of the major fibronectin receptors. Therefore, if cytohesin function is required for integrin β1 recycling, then adhesion to fibronectin will require cytohesin function. We used SecinH3, a recently identified cytohesin inhibitor, to determine if cytohesin function is required for adhesion to fibronectin (24). The importance of cytohesin function on integrin mediated cell to substratum adhesion was first examined by plating Hela cells on different concentrations of fibronectin in the
presence or absence of 15 μM SecinH3. We found that cell adhesion was significantly decreased in SecinH3-treated cells (Fig. 1A). We next investigated which cytohesin member is the major GEF acting in this process. We reasoned that cytohesin 2/ARNO and cytohesin 3/GRP1 are attractive candidates because only ARNO and GRP1 are ubiquitously expressed while cytohesin 1 and 4 are primarily leukocyte specific (25). We therefore transfected myc-tagged ARNO or flag-tagged GRP1 into Hela cells and expected that overexpression of the relevant cytohesin should enhance the adhesion in the same assay. Interestingly, cell adhesion to fibronectin was enhanced in ARNO transfected cells but repressed in GRP1 transfected cells (Fig. 1B). This unexpected inhibition may indicate that the extra GRP1 acts in dominant negative fashion by either competing with endogenous ARNO, or by sequestering necessary downstream components.

We confirmed the differential effects of ARNO and GRP1 on cell adhesion by reducing the expression of each GEF using siRNAs. Transfection of Hela, MCF-7 or MDCK cells with siRNAs targeting ARNO or GRP1 reduces the expression level of mRNA for these proteins (Fig. S1). Cell adhesion was inhibited in cells with reduced ARNO expression as we expected. Strikingly, we also observed that adhesion was enhanced in cells with reduced GRP1 expression (Fig. 1C). This observation may indicate either that components sequestered by GRP1 are released and therefore more are available to ARNO or that ARNO expression is increased to compensate for GRP1 knockdown. Either way, these data suggest that there is more surface integrin β1 integrin or increase integrin activity in the cells with reduced GRP1 expression.

Cells with enhanced surface levels or activity of integrin β1 should not only adhere more tightly to fibronectin, but should also spread more rapidly when plated on fibronectin. Conversely, cells with reduced levels or activity of surface integrin should show impaired spreading on fibronectin. Therefore, we confirmed the results shown in figure 1A, B and C by investigating the spreading of MCF7 cells treated with siRNAs to reduce expression of ARNO or GRP1. Consistent with the results of the adhesion experiments, we found that spreading was impaired in cells transfected with siRNAs targeting ARNO and enhanced in cells transfected with siRNAs targeting GRP1 (Fig. 1D). These experiments also confirm that ARNO and GRP1 have opposing effects on adhesion in multiple cell types.

Enhanced adhesion to fibronectin can be due either to increased levels of surface integrin or to increased activity of the integrins present on the surface (inside-out signaling). Previous work demonstrated that ARF6 regulates the endocytosis and recycling of β1 integrin while cytohesin1 has also been
implicated in inside-out signaling (17, 26). Therefore ARNO could be enhancing adhesion through either pathway. To distinguish between effects on integrin trafficking and effects on integrin activity we directly measured the surface levels of β1 integrin.

Cell surface levels of β1 integrin in cells transfected with siRNAs targeting ARNO or GRP1 were determined by labeling of the cell surface integrin with antibody to integrin β1 followed by precipitation of the antibody-bound protein. Surface integrin levels were reduced in the ARNO knockdown MCF-7 cells (Fig. 2A, B). Next, we performed the same assay in Hela cells overexpressing Myc-ARNO or Flag-GRP1. Surface integrin levels were elevated in the ARNO expressing cells, and reduced in the GRP1 expressing cells (Fig. 2C, D). We confirmed that Hela cells overexpressing myc-ARNO or flag-GRP1 have altered levels of surface integrin using flow cytometry. These cells were fixed and stained with labeled antibody directed against β1 integrin. Cell surface integrin levels measured by flow cytometry were also enhanced in cells overexpressing ARNO and reduced in cells overexpressing GRP1 (Fig. 2E). These data suggest that ARNO modulates adhesion by regulating cell surface levels of integrin and not by altering integrin activity. Given the enhanced adhesion seen in cells treated with GRP1 siRNA (Fig.1), we expected to see elevated levels of surface integrin in these cells. However we did not observe a significant change in the surface levels of β1 integrin in these cells. This assay may not be sensitive enough to see a modest change in cell surface integrin. Alternatively these data may suggest that GRP1 has an inhibitory effect on inside-out signaling. Given the reduced cell-surface β1 integrin levels seen in cells overexpressing flag-GRP1 we favor former explanation.

β1 integrin recycling requires cytohesins- Surface levels of adhesion proteins are balanced by internalization and recycling. Cell surface levels of integrins are increased during cell migration (27). Both integrin β1 internalization and recycling are under control of ARF6 (17, 21). Internalization of integrin β1 requires activation of ARF6 by the GEF Brag2 (22). However, the GEF that activates ARF6 during the recycling of integrin β1 remains unclear. Figures 1 and 2 demonstrate that altering cytohesin expression modulates cell surface levels of β1 integrin. Therefore, we directly tested whether cytohesins are required for integrin recycling. First, an antibody to integrin β1 was bound to cell surface integrins. The cells were then incubated to allow internalization of the antibody bound proteins. Any antibody remaining on the cell surface was removed, and the cells were triggered to recycle internalized antibody bound proteins to the cell surface. The amount of recycled antibody on the cell surface was determined by
fluorescent staining. Alternatively, immunoprecipitation was used to determine the levels of antibody bound proteins retained within the cells (17). We found that integrin β1 recycling in MCF-7 cells follows a timecourse similar to that previously seen in Hela cells (Fig. S1). (17). Most of the integrins were recycled back to the cell surface within 5min of treatment with 20% FBS, and were subsequently re-internalized within 15min. (Fig. S1). We directly tested the hypothesis that cytohesins are required in integrin β1 recycling. Consistent with adhesion results, integrin β1 recycling was reduced when cytohesin function was impaired by treatment of the cells with SecinH3. Most of integrin β1 was still inside SecinH3 treated cells after 5min stimulation (Fig. 3A), and almost none of previously internalized integrin β1 was on the cell surface after the stimulation with 20% FBS, in contrast to the untreated cells (Fig. 3B).

Only ARNO is required for integrin β1 recycling- Figures 1 and 2 suggest that ARNO but not GRP1 is required for integrin β1 recycling. Therefore, we measured β1 integrin recycling in MCF-7 cells transfected with siRNAs targeting ARNO or GRP1. Integrin β1 recycling was significantly inhibited in cells transfected with an siRNA targeting ARNO (Fig. 4). Cells transfected with an siRNA directed against GRP1, on the other hand, showed no impairment of integrin β1 recycling at a 5 minute timepoint. Most of integrin β1 was recycled back to the cell surface in the control and GRP1 siRNA treated cells, but ARNO-siRNA treated cells still had not recycled internal integrin β1 after 5 minutes of stimulation with 20% FBS (Fig. 4A and 4C). Figure 1C suggests that the GRP1 knockdown should have increased recycling, but we did not observe this effect when recycling was directly tested. The reason may be differences in sensitivity of the two assays or the different cell types used in the two experiments.

**ARNO and GRP1 knockdown have opposing effects on cell migration-** Since integrin mediated adhesion has been shown to promote cell migration, we tested the effect of cytohesin knockdown on migration. Migration was measured by using the Oris migration chamber. In this system, cells are grown around a silicon plug. The plug is then removed and the cells migrate into the area vacated by the plug. We seeded MDCK cells transfected with siRNAs targeting ARNO or GRP1 into the chambers. After an overnight recovery, the stopper was pulled out to initiate the cell migration, and the cells allowed to migrate into the open area overnight. As expected, cell migration was reduced in cells treated with siRNA targeting ARNO, but enhanced in cells transfected with siRNA directed against GRP1. Our data all suggest that ARNO/cytohesin 2 regulates integrin β1
recycling and that GRP1/cytohesin 3 does not regulate this process.

*Localization of cytohesins in spreading cells*- One explanation for the fact that cells express multiple GEFs is that the different GEFs act at different subcellular locations. Therefore we investigated the localization of cytohesin 2 and 3 in cells spreading on fibronectin. MCF-7 cells expressing either flag-tagged GRP1 or myc-tagged ARNO were spread on fibronectin for 30 minutes then fixed and the cytohesin localization determined by immunofluorescence. Cytohesin 2/ARNO was found at both at the edges and throughout the interior of spreading cells. Cytohesin 3/GRP1, on the other hand, was almost exclusively located at the very peripheral edge of spreading MCF-7 cells (Fig. 6A). We confirmed the differential localization of cytohesin 2 and 3 using deconvolution microscopy of MCF-7 cells co-expressing both cytohesins. The deconvolved images confirm that in spreading cells cytohesin 3 is exclusively located at the cell periphery, while cytohesin 2 is present at the periphery and at more interior regions (Fig. 6B). Furthermore projection along the Z-axis of the deconvolved stack shows that cytohesin 3 is restricted to the most basal level of the cell, while cytohesin 2 is also present in more apical regions (Fig. 6C).

**Discussion**

Cellular migration is a well-coordinated process that requires altered cell shape and polarity (20). Recent studies have shown that small GTPases of the ADP-ribosylation Factor (ARF) family are involved in these processes (28). Furthermore, it has been demonstrated that ARF6 regulates endocytosis and recycling of adhesion proteins, which is one mechanism that can facilitate cell migration (17, 21). In this study, we demonstrated that integrin β1 recycling is regulated by cytohesin 2/ARNO. We also found that two similar ARF6 GEFs, cytohesin 2/ARNO and cytohesin 3/GRP1, have different functions in cell adhesion, spreading and migration. ARNO knockdown reduces adhesion, spreading and migration, while GRP1 knockdown enhances adhesion, spreading and migration. Additionally we found that ARNO but not GRP1 is required for β1 integrin recycling.

**Integrin β1 recycling pathway**

Integrins are continuously cycling by endocytosis and reinsertion into the plasma membrane (29, 30). This trafficking mechanism regulates cell migration. Integrin is redistributed from the trailing edge to the leading edge of migrating cells (31). ARF6 is known to regulate both integrin β1 endocytosis and recycling (17, 21).
The itinerary taken by ARF6 dependant cargo has been elucidated. These cargo proteins, such as β1 integrin, are internalized by clathrin independent endocytosis. They then enter the early endosome where they meet up with cargo that was internalized in a clathrin dependent manner. Recycling cargo then travel to the Rab11 positive endosomal recycling compartment. ARF6 dependent cargo is then returned to the plasma membrane via a tubular recycling endosomes that align along microtubules. Both the internalization and recycling steps of this pathway require ARF6 activity. (6)

Despite the existence of 15 ARF GEFs, there are only 6 ARFs expressed in mammalian cells (10, 32). This suggests that ARF activation is regulated by different GEFs in different signaling pathways. The 15 Sec7 family members are divided into 5 classes: GBF/BIG, Cytohesins, EFA6, BRAGs and Fbox (9).

Previous research showed that Brag2 is localized to plasma membrane and activates ARF6 to regulate integrin β1 endocytosis (22). It also has been demonstrated that ACAP1, a GAP for ARF6, is involved in recycling of this protein (21). However, the ARF6 GEF involved in integrin β1 recycling remained unknown. Cytohesin 2/ARNO has been shown to stimulate epithelial cell migration, and cytohesin 3/GRP1 induces actin cytoskeleton rearrangement (5, 33). In addition, recycling of adhesion proteins is one of the mechanisms to regulate cell migration. Therefore, we tested cytohesins as promising candidates and identified ARNO as the ARF GEF required for integrin β1 recycling.

**Different functions of ARNO and GRP1 in cell adhesion, spreading and migration**

The cytohesin family has 4 isoforms: cytohesin 1, cytohesin 2/ARNO, cytohesin 3/GRP1 and cytohesin 4. They share similar sizes, extensively conserved sequences, and a common domain structure: coiled coil, sec7, PH and basic domain (9). Among the members, ARNO and GRP1 are ubiquitously expressed and they are very closely related (80% identity) (25). Previous research demonstrated that knockdown of either ARNO or GRP1 has similar effects on insulin signaling (24). Here, we demonstrated different functions of ARNO and GRP1 in cell adhesion, spreading and migration. ARNO knockdown reduced cell adhesion, spreading and migration, while GRP1 knockdown had opposing effects on these processes. To our knowledge, this is the first demonstration of differential actions of ARNO and GRP1.

In addition to the different actions of ARNO and GRP1 in integrin β1 recycling, we
find that these proteins are uniquely localized in spreading cells. Cytohesin 3/GRP1 is exclusively located in the most peripheral and basal regions of spreading MCF-7 cells. This localization suggests that GRP1 may regulate the protrusive actin rearrangements that are occurring in these locations as the cells spread. Cytohesin 2/ARNO is co-localized with cytohesin 3/GRP1 in these protrusive regions. In addition cytohesin 2/ARNO is located throughout the interior of the cell. It is likely that some of this more centrally located ARNO is promoting the recycling of integrin β1 from endosomal structures to the cell surface.

While ARNO and GRP1 are 80% identical overall, their coiled-coil domains are only 50% identical. Therefore, one explanation of the different sub-cellular locations of ARNO and GRP1 is that they may interact with different partners via their coiled-coil domains. Several scaffolding proteins have been identified that bind to coiled-coil domain of ARNO and/or GRP1. GRASP/Tamalin and CYBR/CASP/CyTip can bind both ARNO and GRP1 (34-36). IPCEF interacts with both ARNO and GRP1 by 2-hybrid although only the interaction with ARNO was confirmed with recombinant proteins (37). GRSP1 interacts with GRP1 and it is not known whether this protein can also bind to ARNO (38). It has been shown that these proteins co-localize with cytohesins and modify their ARF6 activating activity (37). Therefore, endogenous ARNO and GRP1 may be bound via their coiled-coil domains to different scaffolding proteins and thereby are recruited to different places.

An possible additional explanation for the different actions of ARNO and GRP1 is the presence of a protein kinase C (PKC) site in the polybasic domain of ARNO that is absent in GRP1 (3). The cytohesin polybasic domain is an intramolecular autoinhibitory domain (39). The polybasic domain and the linker region between the Sec7 and PH domains act as a pseudosubstrate to occlude the active site. This autoinhibition is relieved either by binding of active ARF6 to the PH domain or by phosphorylation of polybasic domain. These alterations disrupt the pseudosubstrate interactions and allow the catalytic Sec7 domain to interact with and activate ARFs (39). PKC activation in ARNO expressing Hela cells produces extensive ruffling and actin rearrangement (3). Integrin β1 recycling may depend upon an activation of PKC that GRP1 cannot respond to. Thus, future investigation into the mechanisms underlying the differing actions of ARNO and GRP1 during integrin β1 recycling will likely provide a better understanding of how migration is regulated by ARF. Such studies will also help to illuminate why ARF6 is subject to regulation by a large number of GEF proteins.
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FIGURE LEGENDS

Figure 1. Cytohesin function during cell adhesion to fibronectin. (A) Cytohesin function is required for cell adhesion. Hela cells were incubated in the presence or absence of 15μM SecinH3. Cells were non-enzymatically harvested and allowed to bind to different concentrations of fibronectin for 60 minutes. Cells were washed, fixed and stained with crystal violet. The stain was extracted into 1% Deoxycholate and OD₅₉₅ determined. (B) ARNO expression enhances cell adhesion to fibronectin, while cell adhesion is repressed by GRP1 expression. Myc-tagged ARNO or Flag-tagged GRP1 was expressed in Hela cells. Adhesion to fibronectin was performed as described in (A). Western blot of cells used in the assay is shown. (C) ARNO knockdown reduces cell adhesion, while GRP1 knockdown enhances cell adhesion. Hela cells were transfected with siRNAs targeting either ARNO or GRP1 as described in Experimental Procedures, and the adhesion assay performed. Data shown (A, B and C) are mean ± standard deviation of triplicate samples and are representative of 3 separate experiments. (D) Effect of cytohesin knockdown on the spreading of MCF-7 cells. MCF-7 cells were transfected with siRNAs targeting either ARNO or GRP1 and spread on fibronectin as described in Experimental Procedures. Spread cell surface area was measured using Image J. Spreading of each sample was normalized to the spreading of cells treated with control siRNAs. Data shown (D) are mean ± standard deviation of seven cells and are representative of 3 separate experiments. (E) Images of the spreading cells analyzed in D. Bar = 25 μM. All data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.

Figure 2. Cell surface expression of integrin β1 in cells with altered levels of cytohesin expression
(A) Surface integrin β1 is reduced in MCF-7 cells with reduced ARNO expression. Surface integrin levels of cells transfected with siRNAs targeting either ARNO or GRP1 were determined by immunoprecipitation of the cell surface integrin labeled with integrin β1 specific antibody (TS2/16). (B) The percent of surface integrin of each cytohesin knockdown cell was determined by comparison to surface integrin expression of control knockdown cells. (C) Cell surface integrin β1 expression is enhanced in Myc-ARNO expressing Hela cells while reduced in Flag-GRP1 expressing Hela Cells. (D) The percent of surface integrin of each cytohesin expressing cell was determined as described for (B). Data shown are mean ± standard error of 3 (knockdowns) or 5 (overexpression) separate experiments. Asterisk indicates P<0.015; double asterisk indicates P<0.01.
Cell surface levels of β1 integrin in Hela cells overexpressing GFP, GFP + ARNO, or GFP + GRP1 were measured by flow cytometry. Cells were stained with AlexaFluor 647 conjugated anti β1 integrin antibody and the median AlexaFluor 647 fluorescence of GFP positive cells determined. Data shown are mean ± standard deviation of median fluorescent intensity from three independent transfections. A total of 10,000 cells were analyzed for each sample. Asterisk indicates P<0.05; double asterisk indicates P<0.01.

Figure 3. Integrin β1 recycling requires cytohesins. (A) Integrin β1 is retained intracellularly in cells treated with SecinH3. Cell surface integrins were labeled with antibody to β1 integrin, and the integrin was internalized as described in Experimental Procedures. Recycling was initiated in the presence or absence of 15 μM SecinH3. Internal integrin was isolated as described in Experimental Procedures and was quantitated by Western Blot. (B) Surface and Total integrin β1 was visualized by Immunofluorescence after the integrin β1 recycling assay for the cells without or with SecinH3 treatment as described in A. Bar, 25μm

Figure 4. Integrin β1 recycling requires ARNO. (A) Cell surface β1 integrin was labeled with antibody, internalized and recycling initiated in MCF-7 cells transfected with siRNA targeting ARNO or GRP1 as described in Experimental Procedures. Retained internal β1 integrin was isolated and visualized by Western blot. (B) The percent of internal integrin β1 5 min after stimulation was determined by comparison to a 0 min stimulation sample. Data shown are mean ± standard error of >3 separate experiments. Levels of internal integrin β1 at 5 minutes in the ARNO or GRP1 knockdown cells were compared to the levels in control cells using a T-test. Double asterisk indicates P<0.01. (C) Surface and Total integrin β1 was visualized in MCF-7 cells transfected with siRNA targeting ARNO or GRP1 after various times of recycling. Bar, 25μm

Figure 5. ARNO and GRP1 knockdown have opposing effects on cell migration. (A) ARNO or GRP1 levels in MDCK cells were reduced by transfection of siRNA for 2 days, and cell migration measured using the Oris migration chamber as described in Experimental Procedures. Images of each well were taken. (B) Cell migration was quantitated by measuring the percent of the starting open area covered by the migrating cells. Data shown are mean ± standard error of 7 separate
experiments. Migration of the knockdown cells was compared to the migration of control cells using a T-test. Asterisk indicates P<0.05; double asterisk indicates P<0.01. Bar, 500μm

**Figure 6.** Differential localization of cytohesin 2 and 3 in spreading cells. (A) MCF-7 cells were transfected with Flag-cytohesin 3 or myc-cytohesin 2 and spread on fibronectin-coated coverslips for 30 minutes. The cells were then stained with rhodamine-phalloidin to stain f-actin and either FITC-conjugated M2 anti-flag or AlexaFluor 488-conjugated 9e10 anti-myc. In the merged image the cytohesin is pseudocolored green and the palloidin is pseudocolored red. Bar, 10μm. (B) MCF-7 cells were cotransfected with Flag-cytohesin 3 and myc-cytohesin2, spread on fibronectin and fixed as above. The cells were then stained with FITC-conjugated M2 anti-flag and biotinylated 9e10 anti-myc, followed by staining with AlexaFluor 546-conjugated streptavidin. A z-stack through the entire depth of the cell was collected and analyzed by deconvolution as described in Experimental Procedures. A maximum projection along the Z-axis is shown. In the merged image cytohesin 3 is pseudocolored green and cytohesin 2 is pseudocolored red. Bar, 10μm. (C) A maximum projection through the X-axis of the deconvolved stack in B is shown in order to visualize the location of cytohesin 2 and 3 in the Z-dimension. Bar, 10μm.
Figure 1

A

B

C

D

E

Cells bound (A. U.)

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1

0

2

4

6

8

10

[Fibronectin] (µg/ml)

Control ARNO GRP1

**

SecinH3

***

Flag-GRP1

Myc-ARNO

GAPDH

Control ARNO GRP1

% Cell Spreading

0

20

40

60

80

100

120

140

160

180

200

Control

ARNO

GRP1

siRNA

**

siRNA

Control

ARNO

GRP1

siRNA

Flag-GRP1

Myc-ARNO

GAPDH

Control ARNO GRP1

% Cell Spreading

0

20

40

60

80

100

120

140

160

180

200

Control

ARNO

GRP1

siRNA

**
Figure 2

A

Surface-B1 Integrin
GAPDH
siRNA | Control | ARNO | GRP1

B

% Surface Integrin

Control | ARNO | GRP1

C

Surface-B1 Integrin
Myc-ARNO
Flag-GRP1
GAPDH
Control | ARNO | GRP1

D

% Surface Integrin

Control | ARNO | GRP1

E

Median Surface Integrin level

Control | ARNO

Control | GRP1
Figure 3

A

|       | Internal-B1 Integrin | GAPDH |
|-------|----------------------|-------|
| SecinH3 | –   | –   | +    | +    |
| Recycling Time (Min.) | 0   | 5   | 0    | 5    |

B

|       | 0 Min | 5 Min | 0 Min | 5 Min |
|-------|-------|-------|-------|-------|
| Surface B1 integrin AB | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) |
| Total B1 integrin AB | ![Image](image5) | ![Image](image6) | ![Image](image7) | ![Image](image8) |
| SecinH3 | – | – | + | + |
Figure 4

A

Internal B1 Integrin
GAPDH
siRNA Control ARNO GRP1
Recycling Time (Min.) 0 5 0 5 0 5

B

% Internal Integrin

Control ARNO GRP1

C

Surface B1 integrin antibody
Total B1 integrin antibody
siRNA Control Control ARNO ARNO GRP1 GRP1
Figure 5

A

![Image of cell migration experiment](image)

siRNA Control ARNO GRP1

B

![Graph showing migration](image)

% Migration

Control ARNO GRP1

siRNA

**
Figure 6

A  
Cytohesin  f-actin  merge  

Cytohesin-2  

Cytohesin-3  

B  
Cytohesin-3  Cytohesin-2  merge  

C  
Cytohesin-3  
Cytohesin-2  
merge  
