THE PENNSYLVANIA STATE UNIVERSITY

The Graduate School

Department of Biochemistry and Molecular Biology

THE DISTINCT ROLES OF CYTOHESIN 2 AND CYTOHESIN 3 IN CELL ADHESION AND MIGRATION

A Dissertation in

Biochemistry, Microbiology and Molecular Biology

by

Seung Ja Oh

© 2011 Seung Ja Oh

Submitted in Partial Fulfilment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2011
The dissertation of Seung Ja Oh was reviewed and approved* by the following:

Lorraine C. Santy  
Assistant Professor of Biochemistry and Molecular Biology  
Dissertation Advisor  
Chair of Committee

Wendy Hanna-Rose  
Associate Professor of Biochemistry and Molecular Biology

Melissa Rolls  
Assistant Professor of Biochemistry and Molecular Biology

Graham Thomas  
Associate Professor of Biochemistry and Molecular Biology

Okhee Han  
Assistant Professor of Nutritional Sciences

Scott B. Selleck  
Professor of Biochemistry and Molecular Biology  
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

In tissues, epithelial cells are normally stationary; however, the cells become migratory in cases of developmental morphogenesis, wound healing, or metastasis. Cellular migration is a well-coordinated process that requires altered cell shape and polarity. Recent studies showed that these processes involve small GTPases of the ADP-ribosylation Factor (ARF) family. In particular, ARF6 regulates endocytosis and recycling of junction proteins, as well as an actin cytoskeleton assembly during cell migration. A large number of GEFs, which likely regulate ARFs at different locations and during different processes, activate ARF6. However, not all of these GEFs’ functions are defined. This study investigates the roles of cytohesin ARF-GEFs in cell adhesion and migration. First, the study shows that cytohesin 2/ARNO and cytohesin 3/GRP1 have distinct functions for cell adhesion, spreading, and migration. Cytohesin 2/ARNO expression enhances cell adhesion, spreading, and migration while cytohesin 3/GRP1 expression reduces cell adhesion, spreading, and migration. Second, the study also shows that cytohesin 2/ARNO is required for β1 integrin recycling while cytohesin 3/GRP1 is dispensable for this process. Third, the study finds that cytohesin 2/ARNO and cytohesin 3/GRP1 have differential localizations in spreading cells. Cytohesin 2/ARNO localizes at both the periphery and interior regions, while cytohesin 3/GRP1 exclusively locates at the plasma membrane. Since the findings indicate that the closely related cytohesin 2/ARNO and cytohesin 3/GRP1 have differential functions and localizations, the investigation also considers the sequence differences of cytohesin 2/ARNO and cytohesin 3/GRP1 that produce the differential effects. Finally, this research demonstrates that the number of glycine residues (2G or 3G) in the Pleckstrin Homology (PH) domain of cytohesins is the critical difference underlying opposing effects of cytohesin 2/ARNO
and cytohesin 3/GRP1 on cell adhesion, spreading and migration. The glycine residues in the PH domain are a known site for PIP2 and PIP3 bindings, and the PH domain of cytohesin 2/ARNO and cytohesin 3/GRP1 have different binding affinity to PIP2 and PIP3. Based on all data, this study proposes that ARNO and GRP1 recruitment to different membrane domains by their different selectivities for PIP2 and PIP3 is responsible for their differential effects on cell adhesion, spreading, and migration. This is the first demonstration of the unique roles of cytohesin 2/ARNO and cytohesin 3/GRP1.
TABLE OF CONTENTS

LIST OF FIGURES .............................................................................................................ix

ABBREVIATIONS ...............................................................................................................xi

CELL LINES .........................................................................................................................xiii

ACKNOWLEDGMENTS .......................................................................................................xiv

CHAPTER 1: Introduction .................................................................................................1
  Epithelial Cells ..................................................................................................................2
  Cell Migration ..................................................................................................................3
  Various types of cell migration .........................................................................................4
  Integrin ............................................................................................................................5
  Cancer and Metastasis .....................................................................................................7
  ADP-ribosylation Factor 6 (ARF6) ...................................................................................9
  Cytohesin Family ............................................................................................................11
  Aims of Study ..................................................................................................................14
  Hypothesis ......................................................................................................................14
  Specific Aim 1 ................................................................................................................15
  Specific Aim 2 ................................................................................................................15
  Specific Aim 3 ................................................................................................................16

CHAPTER 2: Materials and Methods..............................................................................17
  Cells and Reagents ........................................................................................................18
  siRNA and Plasmids .....................................................................................................18
CHAPTER 3: Differential Effects of Cytohesin 2 and 3 on Cell Adhesion, Spreading and Migration

Rationale....................................................................................................................28
Cytohesins are critical for cell adhesion to fibronectin............................................29
ARNO and GRP1 knockdown induce differential effects on cell spreading ..........33
ARNO and GRP1 knockdown have opposing effects on cell migration ..........33

CHAPTER 4: Determination of Guanine-nucleotide Exchange Factor (GEF) which regulates β1 integrin recycling.................................................................36
CHAPTER 5: Identification of the cytohesin 2/ARNO and cytohesin 3/GRP1 sequence differences that underlie involvement in integrin recycling

CHAPTER 6: Discussion

Differential functions of cytohesin 2/ARNO and cytohesin 3/GRP1 in cell adhesion, spreading and migration
Integrin Traffic........................................................................................................67

Counteracting function of GRP1.................................................................71

CHAPTER 7: Future direction..........................................................75

Rescue of integrin recycling ...................................................................76

Experimental approach............................................................................76

Investigation of co-localization of cytohesin 2/ARNO and cytohesin 3/GRP1 with PIP2
and PIP3.................................................................................................78

Experimental approach.........................................................................79

E-cadherin recycling study.......................................................................80

Experimental approach.........................................................................81

REFERENCES...............................................................................................84

APPENDIX A: siRNA knockdown efficiency........................................92

APPENDIX B: Source of contents..........................................................93
LIST OF FIGURES

Figure 1.1 The metastatic process ................................................................. 8
Figure 1.2 The exchange cycle of GTPases ..................................................... 10
Figure 1.3 The Sec7 Family of ARF GEFs ..................................................... 13
Figure 2.1 Surface Antibody Binding Recycling Assay .................................... 22
Figure 2.2 Schematic of cell migration measurement ..................................... 24
Figure 3.1 Cytohesin function is required for cell adhesion .............................. 30
Figure 3.2 ARNO expression enhances cell adhesion to fibronectin, while cell adhesion is repressed by GRP1 expression ................................................................. 31
Figure 3.3 ARNO knockdown reduces cell adhesion, while GRP1 knockdown enhances cell Adhesion ........................................................................................................... 32
Figure 3.4 Effect of cytohesin knockdown on the spreading of MCF-7 cells ........ 34
Figure 3.5 ARNO and GRP1 knockdown have opposing effects on cell migration .... 35
Figure 4.1 Cell surface expression of β1 integrin in cells with altered levels of cytohesin Expression .................................................................................................................. 39
Figure 4.2 Cell surface levels of β1 integrin in Hela cells overexpressing GFP, GFP + ARNO, or GFP + GRP1 measured by flow cytometry ...................................................... 40
Figure 4.3 β1 integrin recycling is detected by the surface antibody binding in MCF-7 cells .... 42
Figure 4.4 β1 integrin recycling requires cytohesins. (A) β1 integrin is retained intracellularly in cells treated with SecinH3 ................................................................. 43
Figure 4.5 β1 integrin recycling requires ARNO .............................................. 45
Figure 4.6 Differential localization of cytohesin 2 and 3 in spreading cells ......... 46
Figure 4.7  Differential localization of cytohesin 2 and 3 in the Z-dimension..........................47
Figure 5.1  Constructs of domain swapped cytohesin 2/ ARNO and cytohesin 3/ GRP1...........51
Figure 5.2  ARNO with GRP1 PHPB expression does not enhance cell adhesion....................53
Figure 5.3  Constructs of domain swapped ARNO and GRP1................................................54
Figure 5.4  ARNO with GRP1 PH expression reduces cell adhesion.........................................56
Figure 5.5  2G ARNO expression reduces cell adhesion to fibronectin while wild type ARNO (3G) expression enhances cell adhesion...............................................................58
Figure 5.6  Effect of 2G ARNO or 3G GRP1 expression on the spreading of MCF-7 cells........59
Figure 5.7  Similar localization of 2G ARNO and wild type GRP1 in spreading cells.............60
Figure 5.8  Differential localization of 3G GRP1 and wild type GRP1 in spreading cells........61
Figure 6.1  Model for β1 integrin recycling.................................................................69
Figure 6.2  ARF6 signaling pathways...........................................................................72
Figure 6.3  Model for counteracting function of GRP1.....................................................73
Figure 7.1  E-cadherin Recycling was detected by the surface biotin labeling technique and the Calcium-Switch Assay..................................................................................82
Figure 7.2  E-cadherin recycling requires cytohesins..........................................................83
Figure A-1  Transfection of siRNA reduces expression of cytohesin 2/ARNO and cytohesin 3/Grp1........................................................................................................92
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF6</td>
<td>ADP-ribosylation Factor 6</td>
</tr>
<tr>
<td>ARNO</td>
<td>ARF Nucleotide binding site opener</td>
</tr>
<tr>
<td>CBP</td>
<td>Cytohesin-binding Protein</td>
</tr>
<tr>
<td>CDE</td>
<td>Clathrin-dependent Endocytosis</td>
</tr>
<tr>
<td>CIE</td>
<td>Clathrin-independent Endocytosis</td>
</tr>
<tr>
<td>CYTIP</td>
<td>Cytohesin 1 Interacting Protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial Growth Factor Receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial Mesenchymal Transition</td>
</tr>
<tr>
<td>ER</td>
<td>Early Endosome</td>
</tr>
<tr>
<td>ERC</td>
<td>Endocytic Recycling Compartment</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Factor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GRASP</td>
<td>GRP1-Associated Scaffolding Protein</td>
</tr>
<tr>
<td>GRP1</td>
<td>General Receptor for Phosphoinositides-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth factor</td>
</tr>
<tr>
<td>IPCEF</td>
<td>Interacting Protein for Cytohesin Exchange Factor</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non Essential Amino Acids</td>
</tr>
<tr>
<td>PB</td>
<td>Polybasic</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP5K</td>
<td>Phosphatidyl 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-diphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>PtdIns (4,5)P2</td>
<td>Phosphatidylinositol 4,5-diphosphate</td>
</tr>
<tr>
<td>PtdIns (3,4,5)P3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras-like proteins in brain</td>
</tr>
<tr>
<td>Re</td>
<td>Recycling Endosome</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>w/</td>
<td>with</td>
</tr>
</tbody>
</table>
**CELL LINES**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks (human cervical cancer cell line)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7 (human breast cancer cell line)</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Lorraine C. Santy, for giving me a wonderful opportunity to work in her lab and for providing guidance to complete my PhD studies. I especially thank her for the 5 years of support that enabled me to understand science, develop the problem solving skills needed to complete this dissertation’s work. She didn’t only teach me scientific knowledge but also how to become a good mentor. She was extremely patient with me whenever I struggled with research, she waited until I understood and solved the problem while she gave generous and pin-point guidance. She is a great scientist and warm mentor.

I also would like to thank my committee members, Dr. Hanna-Rose, Dr. Rolls, Dr. Han and Dr. Thomas. They provided me with time to discuss my project and gave me many suggestions to develop my research forward. I thank my wonderful lab mates. Myriam was a very special friend. We started the graduate studies together in the same program and lab. We shared many special moments together. When I was struggling, she always tried to cheer me up. I hoped that I could be such a friend to Myriam. Heather was a very insightful friend. She challenged me with numerous topics to discuss, and I really enjoyed them. She also influenced me to make healthy food choices, and I have her to thank for that. I especially thank Joe for his patience in proofreading. Thank you also to David White and Katie McShea for being helpful as I settled in the Santy lab.

I particularly thank all my family members. Thank Jaekyun, my husband, for being patient and supporting me along those challenging 5 years. You are my best friend. Thank Chloe (aka Jiho), my daughter. You are the best present I ever had in my life. Thank you for your smile. I really love you. Thank you father (Kongmyung) and mother (Jongim), and my sisters...
(Seungkung and Seungjoo) for supporting me to be who I am both personally and professionally.

I am very lucky to have you all in my life.

Finally, thank GOD. You are the reason for me to be in this world.
CHAPTER 1
INTRODUCTION
Epithelial Cells

Epithelial cells are usually non-motile; however, they become migratory in metastasis. More than 80% of cancers arise from epithelial cells [1]. Therefore, understanding what epithelia are and how epithelial cells acquire motility is important to prevent or cure cancer. Epithelial cells grow as sheets; the structure of which is dictated by cell-cell and cell-matrix adhesions. There are various epithelial junctions which maintain cell-cell and cell-matrix adhesions: tight junctions, adherens junctions, desmosomes, gap junctions and hemidesmosomes. Tight junctions form barriers between epithelia to inhibit leakage of water and solutes by sealing the cells together. Epithelial cells line most organs; in particular, cells lining the small intestine maintain the contents of the lumen inside by forming tight junctions [2]. Adherens junctions, known to provide strong mechanical attachments between adjacent cells by connecting actin filaments, can coordinate cell contraction, which can help morphogenesis. Desmosome junctions hold neighboring cells together while hemidesmosomes mediate cell-extracellular matrix (ECM) bindings. Also, gap junctions allow cells to communicate with others by permitting passage of small molecules. These junctions maintain tissue integrity by mediating cell-cell and cell-substratum adhesions [2].

Cell-cell and cell-matrix adhesions are important in many biological processes. They not only have physical adhesive functions but also regulate signal transduction pathways. Crucial biological processes, such as developmental morphogenesis, require alteration of expression and function of cell adhesion proteins. Cadherin, a tight junction protein, shown to be an important regulator of formation and dissociation of epithelial cell sheets or clustering, affects gastrulation, neurulation and somite formation, when changes of expression patterns of this protein occur [2].
Of particular interest is that tumor progression, an undesired biological process, also utilizes these mechanisms. In the progression towards tumor malignancy, epithelial cells lose cadherin-mediated cell-cell adhesion, and the cell body changes shape and stiffness by modulating cell adhesions to interact with the surrounding environment [3-4].

Cell Migration

In normal circumstances, epithelial cells are non-motile; the cells of epithelial tissues are attached to each other by cell-cell adhesions and further anchored by cell-substratum adhesions [5-6]. However, the cells become migratory during developmental morphogenesis, wound healing and metastasis by extensively remodeling their adhesions [5]. Adhesion remodeling is one mechanism to facilitate cell migration [7]. Epithelial cells utilize two types of migration that differ in terms of adhesion rearrangement. First, cells can migrate as a sheet by maintaining their adherens junctions. The cells utilize this type of migration during wound healing, tissue morphogenesis and development. Alternatively, cells break their adherens junctions and migrate independently as apparent in both normal development and metastasis of epithelial tumors [8-9].

Cell migration is a cyclic process [10]. First, cells respond to stimulation to promote cell migration and polarize to establish the direction of migration. Next, once polarized, cells extend protrusions, actin-rich structures at the leading edge of migrating cells, and stabilize the protrusion by forming new adhesions. Last, cells retract their rear edges, which disassemble adhesions. Protrusion formation and stabilization are very important steps in cell migration. It
has been shown that the formation of integrin-mediated cell adhesion is critical for protrusion formation. Integrins are the major class of receptors mediating cell-substratum adhesions [11]. Activated integrins are preferentially localized at the leading edge of migrating cells where new adhesions form [12]. Expression levels of integrin on cell surface determine types of cell migration.

**Various types of cell migration**

Cancer cells use various migration strategies to move through tissues; as individual cells, “individual cell migration” or as a group, “collective cell migration” [3]. Individual cell migration has two different morphological variants. One is an amoeboid type, and the other is a mesenchymal type. Levels of integrin mediated cell-substratum adhesion and protease production determine the type of individual cell migration [3]. Since the cells in amoeboid migration do not focalize contacts, their morphology is transient, and velocity is higher than mesenchymal migration, consequently, amoeboid migration is less adhesive than mesenchymal migration. Conversely, the cells undergoing mesenchymal migration form focal contacts, and these focal adhesions induce slower migration [3].

Collective cell migration also has two different variants: strand or sheet type and cluster type. In contrast to individual cell migration, collective migration maintains cell-cell adhesion, and cells move as a single functional unit. Generally, leukaemias and lymphomas disseminate as single cells, while epithelial cancers usually use a collective cell migration mechanism [3].

The balance of cell-ECM interaction and cell-cell adhesions determine interconversion between individual cell migration and collective cell migration. Collective cell migration has
both increased cell-ECM and cell-cell adhesions compared to individual cell migration. On the other hand, individual cell migration tends to have weaker cell-substratum adhesion than collective migration and no cell-cell adhesion. Therefore, the mechanism how epithelial cells balance cell-ECM and cell-cell interactions is important to understand cell migration.

Integrins and cadherins are two important classes of junctional proteins involved in the determination of cell migration modes. Integrin mediates cell-substratum adhesion, and cadherin mediates cell-cell adhesion. In collective migration, the cells have higher cadherins expression than cells undergoing individual migration. No cadherin mediated cell-cell adhesion is apparent in individual cell migration. Increasing evidence suggests that crosstalk between integrins and cadherins regulates cell migration levels and modes. E-cadherin is down regulated, and integrin is upregulated during epithelial-mesenchymal transition (EMT) and metastasis [9, 13]. Research has also shown that increased integrin adhesion promotes migration and scattering [14-15], while E-cadherin adhesion reduces lamellipodia, scattering and invasion [16-17]. In addition, forced E-cadherin in metastatic cancer cells is capable of preventing metastasis [13]. Collectively, previous research suggests that increasing surface integrin levels can promote epithelial migration and metastasis.

**Integrins**

Integrins are well studied proteins which are involved in many biological processes including cell migration. Integrins are heterodimeric cell junction proteins that interact with matrix proteins such as fibronectin. Once integrins bind to the matrix, the cytoplasmic domain of
the integrin β subunit binds to the cytoskeleton to maintain strong cell-matrix adhesions. This binding induces integrin clustering and focal adhesion formation between the cell and the extracellular matrix. Integrin to ligand binding can activate signal transduction pathway in the cell. Signaling proteins such as focal adhesion kinase (FAK) are recruited the active integrin cluster and initiate various cell signal transduction pathways. This is referred to as outside-in integrin signaling. In contrast to outside-in integrin signaling, when signal transduction pathways within the cell trigger activation of integrin resulting in integrin to ligand binding, this is called inside-out integrin signaling [2]. Once the cell forms focal adhesions, integrin traffic promotes cell migration by internalizing integrins at the trailing edge of the cell and recycling them to the leading edge [18-19]. A balance between endocytosis and recycling determines cells’ surface levels of integrin, and previous publications show that ARF6 and Rab family members control both the internalization and recycling of β1 integrin [20-22]. Integrin-ECM interaction induces formation of focal contacts in which integrin connect with actin cytoskeleton via the FAK-Src complex. However, internalization of β1 integrin is associated with the disassembly and turnover of focal contacts [7]. During focal contact disassembly, the integrin loses connections to actin, and internalization sites become the targets of microtubules. At these sites, Rab5/Rab21, ARF6 and microtubules regulate β1 integrin internalization [22]. Brag2 activates ARF6 to induce β1 integrin endocytosis. Rab5 plays a role in endocytosis and endocytic vesicle movement along microtubules [22]. In the early endosome, Rab21 binds to α subunit of β1 integrin. From the early endosome, β1 integrin travels to Rab 11 positive perinuclear recycling endosome. Finally, Rab11, Rab21 and ARF6 promote long-loop recycling of β1 integrin back to the plasma membrane [23].
Cancer and Metastasis

Cancer currently causes 1 in 4 deaths in the United States [1]. The American Cancer Society reported 1,529,560 new cancer cases and 569,490 deaths by cancers in the United States in 2010. Prostate, lung and bronchus and colorectal cancers were the most common cancers occurring among men, and breast, lung and bronchus and colorectal cancers were three most diagnosed cancers for women in 2010. Although cancer incidence and mortality have declined in recent years, cancer remains the leading cause of death for people younger than 85 [1].

Cancer is a genetic disease from mutations in somatic cells. There are usually two classes of mutation target genes in cancer. One is proto-oncogenes that regulate cell growth and differentiation among normal cells. The other type is tumor suppressor genes that inhibit cancer progression. Tumor suppressor genes usually follow the ‘Two-hit hypothesis’. Since tumor suppressor alleles are recessive, both alleles need to be mutated to be malfunctioned. However, oncogenes do not require mutations in both alleles because their alleles are dominant, so they gain the function even with a single mutation. For cancer development, the cells need mutations in both oncogenes and tumor suppressor genes [2].

Cancer development is a process in which cells are unable to limit cell growth and acquire the ability to evade apoptosis, and sustain angiogenesis. Most human cancers progress into metastatic status when cancer cells leave their original locations and travel into distant organs. The progression of metastasis consists of a series of critical steps (Fig. 1.1). First, cancer cells detach from the primary tumor and invade the basal lamina surrounding the tumor. The migrating cells then enter blood or lymph vessels. Next, the cells exit these vessels and enter a distant organ of the body. Last, the migrant cells acquire the ability to survive and grow in the
secondary site [24]. Regulation of cell adhesions mediates dissemination of cancer cells from the primary tumor and attachment of the cells to the vascular endothelium within a distant organ. Cell adhesions are regulated by junctional proteins, of which E-cadherin and integrin are crucially important to the occurrence of metastasis. Attachment of metastatic cells to new sites occurs through the E-cadherin [25]. And, expression of different subunits of integrin induces or inhibits metastasis [24]. ADP-Ribosylation Factor 6 (ARF6) regulates both integrin and E-cadherin expression on cell surface, thereby regulating cell migration.

**Figure 1.1 The metastatic process**

Cancer cell is disseminated from the primary tumor, and invades the matrix surrounding tumor. It enters blood vessels and travels to a distant organ in the body. The cell exits the vessels and settles down in a new organ by acquiring the ability to survive and grow. Adapted from Chambers et al. (2002) Nat Rev Cancer 2: 563-572
ADP-Ribosylation Factor 6 (ARF6)

ARFs (ARF1-6) are small GTPases of the Ras superfamily and regulators of vesicular transport. Like other small GTPases, ARFs are molecular on/off switches that cycle between active GTP and inactive GDP bound forms. GTPases require accessory proteins to facilitate activation or inactivation. Guanine nucleotide-exchange factors (GEFs) catalyze activation of GTPases, and inactivation requires GTPase activating proteins (GAPs) (Fig. 1.2) [26-27]. Increasing evidence indicates that ARFs are activated or inactivated by one or more ARFs specific GEFs or GAPs [28-29].

Six known ARF isoforms constitute three classes: Class I ARFs (ARFs 1-3), localized on Golgi, regulates the assembly of vesicle coat complexes in the secretory pathways and at endosome. Class II ARFs (ARFs 4 and 5) are still poorly understood. The only member of Class III, ARF6, localized at the cell periphery, regulates trafficking between the plasma membrane and endosomal system [30]. The N-terminus of ARF6 associates with membranes via a myristoyl group [27, 31-32]. The inactive form of ARF6, ARF6-GDP, associates with intracellular vesicular compartments [28, 31, 33]. Once activated, ARF6, recruited to the plasma membrane, regulates endocytosis, exocytosis and endosomal traffic [28, 33-34].

ARF6 regulates assembly of the cytoskeleton and cell motility [35-38], and ARF6 activation induces rearrangement of cortical actin cytoskeleton and ruffling [33, 38]. Cytohesin 2/ ARNO, an ARF6-GEF, promotes cell migration by activating ARF6, and subsequently active ARF6 promotes activation of Rac 1 [38]. Since cell migration is a well coordinated process that requires altered cell shape and polarity, cells should modulate cell-cell and cell-substratum adhesions for cell migration. Endocytosis or recycling of junction proteins is one mechanism to
alter stable cell-cell and cell-substratum adhesions. ARF6 regulates the endocytosis and recycling of junction proteins such as 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 [39].

Figure 1.2 The exchange cycle of GTPases

GTPases are activated by assistance of guanine-nucleotide exchange factors (the GEFs) and inactivated by GTPase activating proteins (the GAPs). Once GTPases are activated by the GEF, they turn on the downstream signal transduction by binding to effectors.
Cytohesin Family

Guanine nucleotide-exchange factors (GEFs) activate ARFs, and GTPase activating proteins (GAPs) inactivate ARFs [26-27]. 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 [31]. Despite existence of 15 ARF GEFs, cells express only 6 ARFs. This indicates that more than one GEF regulates an ARF and suggests that ARFs use different ARF-GEFs in different signal transduction pathways. The 15 Sec7 family members represent 5 classes: GBF/BIG, Cytohesins, EFA6, BRAGs and Fbox (Fig. 1.3) [31]. Among the 5 classes, cytohesins are the best characterized 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 [28, 31]. Both cytohesin 2/ARNO and cytohesin 3/GRP1 are ubiquitously expressed, but cytohesin 1 and 4 are primarily found in leukocytes [40].

Cytohesins are recruited to the plasma membranes in response to phosphatidylinositol 3 kinase signaling [41-42]. Cytohesin 1, cytohesin 2/ARNO and cytohesin 3/GRP1 have 2 splice forms, with either a diglycine or a tryglycine stretch in the β1/β2 loop of the PH domain [43]. Cytohesin 1 and cytohesin 2/ARNO are predominately in the triglycine isoform, while cytohesin 3/GRP1 and cytohesin 4 are mostly in the diglycine isoform [40]. Intriguingly, research has shown that the diglycine form of cytohesins has a strong selectivity for phosphatidylinositol 3,4,5-triphosphate (PIP3), but the triglycine form has an equal affinity for PIP3 and
phosphatidylinositol 4,5-diphosphate (PIP2) [43]. Cytohesins are localized to membranes by binding to these specific PIPs [26, 38].

Cytohesins regulate actin rearrangements and cell motility [36, 41-42]. Cytohesin 1 regulates the spreading and transendothelial migration of lymphocytes, and cytohesin 2/ARNO induces broad lamellapodia and cell migration in Madin-Darby Canine kidney (MDCK) epithelial cells [38, 44]. Cytohesin 2/ARNO colocalizes with ARF6 at the plasma membrane. Cytohesin 2/ARNO is recruited to the plasma membrane in response to PIP3 production by PI-3 kinase and induces rearrangements of cortical actin cytoskeleton and cell migration [28, 36, 38, 45-46]. Like all cytohesins, cytohesin 2/ARNO contains coiled-coil, sec7, PH and polybasic domains. The N-terminal coiled-coil domain of cytohesin 2/ ARNO mediates homodimerization and interactions with other proteins. Sec 7 is the catalytic domain which can activates ARFs. The PH domain mediates membrane localization by binding to phosphoinositides such as PIP2 and PIP3 [26, 38]. The polybasic domain represents an intramolecular autoinhibitory domain [47].

Cytohesin 3/GRP1 is a less studied protein than cytohesin 2/ARNO. Even though cytohesin 3/GRP1 and cytohesin 2/ARNO are very similar proteins, they have some differences. There is no PKC phosphorylation site in the polybasic domain of cytohesin 3/GRP1 [36]. In addition, cytohesin 3/GRP1 and cytohesin 2/ ARNO only share 50% sequence identity in the coiled-coil domain despite of 80% sequence identity overall. Thus, there are variabilities in proteins to bind to the coiled-coil domain of cytohesin 3/GRP1 and cytohesin 2/ARNO.
There are 15 ARF GEFs expressing in mammalian cells. Among them, cytohesins, EFA6 and BRAGS are GEFs for ARF6. Cytohesins are well studied family of ARF6 GEFs, and have 4 isoforms. They share the same domains: coiled-coil, sec 7, PH and poly basic. Adapted from Casanova (2007) Traffic
Aims of Study

To investigate the involvement of cytohesins in cell migration and adhesion, and to
determine which cytohesins regulate these processes

The focus of this study is to investigate the roles of cytohesin 2 and cytohesin 3 in cell migration
and adhesion, and to determine the sequence differences underlying their distinct functions.
Clear understanding of functional differences between the cytohesins illuminates the mechanism
for extensive regulation of ARF6 in the cell.

Hypotheses

1. Cytohesins are not redundant and have specific roles in adhesion and migration.
2. Cytohesins regulate epithelial cell adhesion by modulating β1 integrin recycling.

Research has shown that the cytohesins modulate migration and adhesion [19, 38, 44, 48].
However, previous studies have not directly compared the actions of different cytohesins. Due to
their high levels of conservation, the general assumption is that cytohesins have redundant
functions. However, all cells express at least two cytohesins, the ubiquitous cytohesin 2/ARNO
and cytohesin 3/GRP1 isoforms. This suggests that cytohesin 2 and cytohesin 3, in fact, have
distinct functions.
**Specific Aim 1:** To test the involvement of individual cytohesins in cell adhesion and migration

There are 15 ARF GEFs in the cells; however, not all of these GEFs’ functions are defined. In the cytohesin family, cytohesin 3/GRP1 is less understood than the very similar protein, cytohesin 2/ ARNO. Extensive implication indicates that cytohesin 2/ ARNO influences inductions of cell motility. Therefore, the current research tests the effects of cytohesin 2/ARNO and cytohesin 3/ GRP1 on cell migration to determine if functional similarities exist between these proteins. Epithelial cells require modulation of cell adhesions during cell migration. Thus, testing of the involvement of cytohesins in regulation of cell adhesion determines whether or not cytohesin 2/ARNO and cytohesin 3/GRP1 have specific roles in this process. This study uses cell adhesion, spreading and migration assays.

**Specific Aim 2:** To determine the guanine-nucleotide exchange factor (the GEF) which regulates β1 integrin recycling

ARF6, known to regulate both endocytosis and recycling of β1 integrin [21, 49], may also regulate cell migration by internalizing integrins at the trailing edge of the cell and recycling integrins to the leading edge [19]. Recent studies demonstrated that Brag2 activates ARF6 to regulate β1 integrin endocytosis while an unknown GEF activates ARF6 to regulate β1 integrin recycling [23]. Researches has shown that Cytohesins, EFA6 and BRAGs activate ARF6 [50]. Among those activators, cytohesins regulate actin rearrangements and cell motility [36, 41-42]. Therefore, the current research tests whether or not either cytohesin 2/ARNO or cytohesin 3/GRP1 regulates β1 integrin recycling. To accomplish this investigation, an antibody binding mediated β1 integrin recycling assay directly tested the GEF involved in β1 integrin recycling.
Specific Aim 3: To determine the sequence differences underlying different functions between cytohesin 2/ARNO and cytohesin 3/GRP1

Surprisingly, cytohesin 2/ARNO and cytohesin 3/GRP1 have opposing effects on cell adhesion, migration, and integrin recycling, despite the fact that these proteins are 80% identical. Analysis of these two proteins determines the sequence differences inducing distinct functions. The current research involves construction of domain swapping fusions of cytohesin 2/ARNO and cytohesin 3/GRP1 to test the effect of these hybrid proteins on adhesion and to determine their localization in spreading cells.
CHAPTER 2
MATERIALS AND METHODS
Cells and reagents

Hela cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Mediatech) 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 GCAAUGGGCAGGAAGAAGU targeting human and canine ARNO, and siRNA duplexes against the sequence GGAGAAGGCCUAAAUAAGA 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. Experiments were carried out 24 hour after transient transfection.
Primer sequences

ARNO_cc_sec7_link_fwd: Fwd primer adds PstI site into ARNO cc sec7 linker
5’ – AAT GAG GGC AGT AAG ACC CTG CAG CGG AAC CGG AAG ATG GC- 3’

ARNO_cc_sec7_link_rev: Rev primer to add PstI site ARNO cc sec7 linker
5’- CAT CTT CCG GTT CCG CTG CAG GGT CTT ACT GCC CTC ATT GG-3’

ARNO_Grp_PB: Primer for adding grp1 PB to arno
5’- GCG CTC GAG TTA TTT TTT ATT GGC AAT CCT TCG TTT TCT CGC TGC CAG CAT CTC ATA GAA GGG-3’

ARNO_sec7_PH_acc_fwd: Fwd primer to add AccIII site to arno sec7 ph linker
5’- GAG CCC TTC AAG ATT CCG GAG GAT GAC GGG AAT GAC C-3’

ARNO_sec7_PH_acc_rev: Rev primer to add AccIII site to arno sec7 ph linker
5’- GGT CAT TCC CGT CAT CCT CCG GAA TCT TGA AGG GCT C-3’

grp_ARNO_PB_antisense: Reverse Complement from grp_ARNO_PB
5’ - GCG CTC GAG TTA GGG CTG CTC CTG CTT CTT CTT GAC TGA AAT CCG CTT CTT CCT CGT TGC CAA CAT GTC ATA GAA GGG-3’

grp_cc_sec7_link_fwd: Primer to add PstI site to grpI cc sec7 linker
5’- TAG AGG AGA GCA AAA CGC TGC AGA GGA ACA AAC AGA TAG CCA TGG-3’

grp_cc_sec7_link_rev: Primer to add PstI site to grpI cc sec7 linker
5’- CTA TCT GTT TGT TCC TCT GCA GCG TTT TGC TCT CCT CTA CGG AAG-3’
grp_sec7_ph_acc_fwd: Fwd primer to add AccI site to grpI sec7 ph linker
5’- GAA CGA GCC ATT TAA GAT TCC GGA GGA CGA CGG GAA CGA CCT G-3’

grp_sec7_ph_acc_rev: Rev primer to add accI site to grp1 sec7 ph linker
5’- CGT TCC CGT CGT CCT CCG GAA TCT TAA ATG GCT CGT TCT TAA TGC-3’

HindIII_myc_5’_ARNO: Primer for HindIII site and myc tag to ARNO
5’- CCG GAA GCT TAT GGA ACA AAA GCT AAT TTC TGA AGA AGA CTT GAT GGA GGA CGG CGT CTA TGA ACC –3’

HindIII_myc_5’_GRP1: Primer for HindIII site and myc tag to GRP1
5’– CCG GAA GCT TAT GGA ACA AAA GCT AAT TTC TGA AGA AGA CTT GAT GGA TGA AGA CGG CGG CGG C –3’

XhoI_3_ARNO: Primer for adding 3’ XhoI site in ARNO
5’- GCG CTC GAG TTA GGG CTG CTC CTG CTT CTT CTT G-3’

XhoI_3_GrpI: Primer for adding XhoI site 3’ in grp1
5’- GCG CTC GAG TTA TTT TTT ATT GGC AAT CCT TCG-3’

GRP1 GGG fwd
5’- CCT GAA GCT GGG GGG CGG CGT GAA GAC CTG GAA GCG G

GRP1 GGG rev
5’- GGT CTT CAC GCG GCC GCC CCC CAG CTT CAG GAG CCA GCC CTC GC-3’

ARNO GG fwd
5’-TGA AGC TGG GCG GCC CGG TGA AGA CGT GGA AGC GGC GCT G-3’
**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. 5x10^4 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 β1 integrin 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 β1 integrin recycling at 37°C (Fig. 2.1) (17). Duplicate samples were fixed at 0 and 5min after the initiation of β1 integrin recycling. One sample was permeabilized by 0.1% triton to observe total
antibody bound integrin while the other sample was left unpermeablized to observe surface β1 integrin. 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.

Figure 2.1 Surface Antibody Binding Recycling Assay

First, β1 integrin specific antibody is applied to the cell surface and then the antibody bound β1 integrin is internalized by incubating at 37°C. Next, surface remained antibody is washed by acidic buffer. Finally, the cells are stimulated to recycle the antibody bound β1 integrin back to the cell surface by treating with 20% FBS.
**Biochemical β1 integrin 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 5 minutes of recycling. Then, the cells were lysed, and the lysate was incubated with protein G-sepharoses to isolate internal antibody bound β1 integrin [21]. Precipitated β1 integrin was analyzed by Western blot for β1 integrin.

**Surface β1 integrin labeling assay**

β1 integrin 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 β1 integrin [21]. Precipitated β1 integrin was analyzed by Western blot for β1 integrin.

**Migration assay**

Cell migration was assessed using Oris migration chambers (Oris™ Universal Cell Migration Assembly Kit: PLATYPUS TECHNOLOGYES, Madison, WI). T23 cells were transfected with siRNAs targeting ARNO or GRP1 for 2 days. Cells (1x10⁵) 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 (Fig. 2.2). The fixed cells were stained with 0.1% crystal violet. Images of each chamber were taken, and cell migration was quantitiated by measuring the remaining cell-free surface area using ImageJ [51].

**Figure 2. 2 Schematic of cell migration measurement**

First, insertion is plugged into 96 wells, and then the cells will be seeded with normal medium and incubated for 24 hours at 37°C. Next, insertion is removed from the wells, and thus the cells starts to migrate. After 24 hours, the cells are fixed and stained.
**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 30min at 30% confluency and then fixed with 4% formaldehyde. The samples were stained with rhodamine-conjugated palloidin and images of each spreading were taken. Cell spreading was quantitated by measuring cell surface area with ImageJ [51].

**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 1mM EGTA/4mM EDTA in PBS for 10 min on ice. Harvested cells were washed with PBS and fixed in cold 4% paraformaldehyde for 15min at 4°C while rotating. Cells were stained with AlexaFluor 647-conjugated anti Integrin β1 (TS2/16) antibody for 1hr 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 0.3 µm 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.
CHAPTER 3

Differential Effects of Cytohesin 2 and 3 on cell adhesion, spreading and migration
Rationale

ARF6 is a small GTPase which functions as a molecular on/off switch. Using its switch function, it controls many different signal transduction pathways. It is known that ARF6 localizes to the plasma membrane and endosomes where it regulates membrane trafficking, actin cytoskeleton remodeling and phosphoinositide metabolism [34]. Although ARF6 function in membrane trafficking is well studied, increasing evidence suggests that ARF6 is also involved in regulation of cell migration. ARF6 is known to be activated by growth factors. For instance, epidermal growth factor (EGF) has been shown to induce ARF6 expression, and EGF receptor (EGFR)-ARF6 signal promotes breast cancer invasion [52-53]. It also has been shown that ARF6 promotes cell migration upon hepatocyte growth factor (HGF)/scattering factor stimulation by activating Rac1 [54]. In addition to regulation of cell migration, ARF6 also modulates endocytosis and recycling of adhesion receptors such as β1 integrin and E-cadherin [21, 49]. Cell adhesion rearrangement is one of the components of cell movement.

To turn signaling pathways on or off, ARF6 requires accessory proteins called GEFs and GAPs. ARF6 is activated by GEFs and then inactivated by GAPs. By cycling between active and inactive forms, ARF6 controls downstream signaling. ARF6 is known to be activated by multiple classes of GEFs; EFAs, Cytohesins and Brags. This indicates that ARF6 is extensively regulated, and each GEF has its own function at particular subcellular locations and during particular signal transduction pathways [31]. Among them, cytohesins are well known regulators which are recruited to the plasma membrane in response to growth factor signaling and promote actin cytoskeleton rearrangement [36, 42, 46]. Cytohesin 2/ ARNO and cytohesin 3/GRP1 are ubiquitously expressed in the cells while cytohesins 1 and 4 are cell type specific. This suggests that most cells will express at least two cytohesin isoforms. However, it is still unclear if they are
redundant or have distinct functions. Cytohesin 2/ARNO has been shown to induce cell motility by activating Rac1 through Dock180/ELMO complex [38]. ARNO expression induces a cell scattering phenotype, similar to what is seen upon HGF treatment. It also has been shown that ARNO regulates cell migration by interacting with focal adhesions [48]. However, the function of the very similar cytohesin 3/GRP1 is still unclear. Therefore, I decided to investigate the involvement of cytohesin 2/ARNO and cytohesin 3/GRP1 in cell adhesion and migration to determine if they have similar or different functions by using cell adhesion, spreading and migration assays. Distinct functions of cytohesin 2/ARNO and cytohesin 3/GRP1 in cell adhesion and migration would support the concept of extensive regulation of ARFs by different GEFs in the different signal transduction pathways.

**Cytohesins are critical for cell adhesion to fibronectin**

Integrin regulates cell migration by altering its expression on the cell surface. β1 integrin is a component of the major fibronectin receptors. Therefore, if cytohesin regulates cell migration via altering cell adhesion, then altering cytohesin function should alter adhesion to fibronectin. I used SecinH3, a recently identified cytohesin inhibitor, to determine if cytohesin function is required for adhesion to fibronectin [55]. The importance of cytohesin function on integrin mediated cell to substratum adhesion was first examined by plating cells on different concentrations of fibronectin in the presence or absence of 15µM SecinH3. I found that cell adhesion was significantly decreased in SecinH3-treated cells (Fig. 3.1).
Figure 3.1 Cytohesin function is required for cell adhesion.

Cells were incubated in the presence or absence of 15µM SecinH3. Cells were nonenzymatically harvested and allowed to bind to different concentrations of fibronectin for 60min. Cells were washed, fixed and stained with crystal violet. The stain was extracted into 1% Deoxycholate and OD$_{595}$ determined. Data shown are mean ± standard deviation of triplicate samples and are representative of 3 separate experiments. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.

I next investigated which cytohesin member is the major GEF acting in this process. I 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 [40]. I 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. 3.2). 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.

![Graph showing cell adhesion to fibronectin](image)

**Figure 3.2 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 Fig 3.1. Western blot of cells used in the assay is shown. Data shown are mean ± standard deviation of triplicate samples and are representative of 3 separate experiments. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.

I 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. A1). Cell adhesion was inhibited in cells with reduced ARNO expression as I expected. Strikingly, I also observed that adhesion was enhanced in cells with reduced GRP1 expression (Fig. 3.3). 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 β1 integrin or increase integrin activity in the cells with reduced GRP1 expression.

![Graph showing cell adhesion](image)

**Figure 3.3** 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 are mean ± standard deviation of triplicate samples and are representative of 3 separate experiments. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.
**ARNO and GRP1 knockdown induce differential effects on cell spreading**

Cells with enhanced surface levels or activity of β1 integrin 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, I confirmed the results shown in figure 3.1, 3.2 and 3.3 by investigating the spreading of MCF-7 cells treated with siRNAs to reduce expression of ARNO or GRP1. Consistent with the results of the adhesion experiments, I found that spreading was impaired in cells transfected with siRNAs targeting ARNO and enhanced in cells transfected with siRNAs targeting GRP1 (Fig. 3.4). These experiments also confirm that ARNO and GRP1 have opposing effects on adhesion in multiple cell types.

**ARNO and GRP1 knockdown have opposing effects on cell migration**

Since integrin mediated adhesion has been shown to promote cell migration, I 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. I 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 were 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 (Fig. 3.5).
Figure 3.4 Effect of cytohesin knockdown on the spreading of MCF-7 cells

(A) 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 are mean ± standard deviation of seven cells and are representative of 3 separate experiments. (B) Images of the spreading cells analyzed in A. Bar = 25 µm. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.

In chapter 3, I showed that ARNO and GRP1 have opposing effects on cell adhesion, spreading and migration. ARNO knockdown reduces cell adhesion, spreading and migration while GRP1 knockdown enhances cell adhesion, spreading and migration. These results suggest that ARNO and GRP1 have distinct functions in cell adhesion and migration. This is the first demonstration of the distinct functions of ARNO and GRP1.
Figure 3.5 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
CHAPTER 4

Determination of Guanine-nucleotide Exchange Factor (GEF) which regulates β1 integrin recycling
Rationale

In chapter 3, I showed that ARNO and GRP1 have distinct functions on cell adhesion, spreading and migration. ARNO knockdown reduces cell adhesion, spreading and migration while GRP1 knockdown enhances cell adhesion, spreading and migration. ARNO has been shown to promote epithelial motility [38]. Since enhanced adhesion is required for cell migration, and ARNO expression enhances cell adhesion, all data shown in chapter 3 are consistent with previous research.

Cell migration can be controlled by modulating the endocytosis and recycling of junctional proteins. Integrin and and E-cadherin are important junction proteins regulating cell migration. There is evidence demonstrating crosstalk between integrin and cadherin-mediated adhesions during cell migration [16, 56]. Loss of E-cadherin function can promote epithelial migration by inducing assembly of integrin-mediated focal adhesions [14-15, 56].

ARF6 has been shown to regulate the endocytosis and recycling of both β1 integrin and E-cadherin [20-22]. While it is known that Brag2, an ARF6-GEF, regulates endocytosis of β1 integrin, the GEF involved in recycling of β1 integrin has yet to be found. Since ARNO is known to promote cell migration by activating ARF6 and Rac1, ARF6 is known to regulate endocytosis and recycling of β1 integrin, and modulation of endocytosis and recycling of junctional proteins is one of the mechanisms to control cell migration, I decided to investigate whether ARNO is acting via integrin recycling to promote cell adhesion and migration. Therefore, I directly tested whether ARNO is involved in recycling of β1 integrin by using the Surface Antibody Binding Recycling Assay described in this chapter. Validating this hypothesis would support the existing link between ARNO function and adhesion during cell migration and also support the concept of distinct functions of GEFs in signal transduction.
**Cell surface β1 integrin**

It was shown in chapter 3 that cytohesin 2/ ARNO enhances cell adhesion while cytohesin 3/ GRP1 reduces cell adhesion. 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 [21, 57]. Therefore, ARNO could be enhancing adhesion through either pathway. To distinguish between effects on integrin trafficking and effects on integrin activity, I 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 β1 integrin followed by precipitation of the antibody-bound protein. Surface integrin levels were reduced in the ARNO knockdown MCF-7 cells (Fig. 4.1 A, B). Next, I 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. 4. 1C, D).
Figure 4. 1 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.
I 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. 4.2). 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), I expected to see elevated levels of surface integrin in these cells. However, I 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 I favor former explanation.

Figure 4. 2 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.
**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 [58]. Both β1 integrin internalization and recycling are under control of ARF6 [20-21]. Internalization of β1 integrin requires activation of ARF6 by the GEF Brag2 [23]. However, the GEF that activates ARF6 during the recycling of β1 integrin remains unclear. Figures 1 and 2 demonstrate that altering cytohesin expression modulates cell surface levels of β1 integrin. Therefore, I directly tested whether cytohesins are required for integrin recycling. First, an antibody to β1 integrin 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, immuno-precipitation was used to determine the levels of antibody bound proteins retained within the cells [21]. I found that β1 integrin recycling in MCF-7 cells follows a timecourse similar to that previously seen in Hela cells (Fig. 4.3). [21]. 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. 4.3). I directly tested the hypothesis that cytohesins are required in β1 integrin recycling. Consistent with adhesion results, β1 integrin recycling was reduced when cytohesin function was impaired by treatment of the cells with SecinH3. Most of β1 integrin was still inside SecinH3 treated cells after 5min stimulation (Fig. 4.4A), and almost none of previously internalized β1 integrin was on the cell surface after the stimulation with 20% FBS, in contrast to the untreated cells (Fig. 4.4B).
Figure 4.3 β1 integrin Recycling is detected by the surface antibody binding in MCF-7 cells.

B1 integrin specific antibody was bound to the surface of serum starved MCF-7 cells, and then labeled integrin was internalized. Residual integrin antibodies remaining on the cell surface were removed with an acid wash, and 20% FBS was used to initiate β1 integrin recycling. (A) 0, 5 and 15min after initiation, recycled integrin antibodies were removed again from the cell surface, and internal integrin was quantitated by Western Blot after immunoprecipitation with protein Gsepharose. (B) After recycling surface or total integrin β1 was detected by immunofluorescent staining of either permiablized or unpermeablized samples. Bar, 25µm
Figure 4. β1 integrin recycling requires cytohesins.

(A) β1 integrin 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 Immunofluoresce after the β1 integrin recycling assay for the cells without or with SecinH3 treatment as described in A. Bar, 25µm
Only ARNO is required for β1 integrin recycling

Data in chapter 3 suggest that ARNO but not GRP1 is required for β1 integrin recycling. Therefore, I 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. 4.5). 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 β1 integrin 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 β1 integrin after 5 minutes of stimulation with 20% FBS (Fig. 4.5 A and 4.5 C). Figure 3.3 suggests that the GRP1 knockdown should have increased recycling, but I 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. My data all suggest that ARNO/cytohesin 2 regulates β1 integrin 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, I 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 immuno-fluorescence. 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. 4.6).
Figure 4. 5 β1 integrin 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 β1 integrin 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 β1 integrin 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 β1 integrin was visualized in MCF-7 cells transfected with siRNA targeting ARNO or GRP1 after various times of recycling. Bar, 25µm
Figure 4. 6 Differential localization of cytohesin 2 and 3 in spreading cells

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.

I 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. 4.7 A). 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. 4.7 B).

In chapter 4, I demonstrated that only cytohesin 2/ARNO, not cytohesin 3/GRP1, regulates β1 integrin recycling. In addition, I also found that cytohesin 2/ ARNO and cytohesin 3/ GRP1 have distinct localizations in spreading cells. These results all support distinct functions of cytohesin 2/ ARNO and cytohesin 3/GRP1 in cell adhesion and migration.
Figure 4.7 Differential localization of cytohesin 2 and 3 in the Z-dimension

(A) MCF-7 cells were cotransfected with Flag-cytohesin 3 and myc-cytohesin2, spread on fibronectin and fixed as Fig. 4.6. 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. (B) 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.
CHAPTER 5

Identification of the cytohesin 2/ARNO and cytohesin 3/GRP1 sequence differences that underlie involvement in integrin recycling
Rationale

As I showed in Chapter 3 and 4, cytohesin 2/ ARNO and cytohesin 3/ GRP1 have distinct functions in cell adhesion, spreading and migration. ARNO enhances cell adhesion, spreading and migration while GRP1 reduces cell adhesion, spreading and migration. I also showed that only ARNO, not GRP1, regulates β1 integrin recycling. Furthermore, I demonstrated that ARNO is localized at both cell periphery and interior regions while GRP1 is only located at the cell periphery. Even though ARNO and GRP1 have distinct functions and localizations, they are very similar proteins that share 80% sequence identity overall. Therefore, I decided to identify the sequence differences that induce the opposite functions of ARNO and GRP1 in cell adhesion and migration by constructing domain swapping hybrid proteins. The result would give us better understanding of how migration is regulated by ARF. This study would also help to illuminate why ARF6 is subject to regulation by a large number of GEF proteins.

Cytohesin 2/ARNO and cytohesin 3/GRP1 Hybrid Constructs

While ARNO and GRP1 are 80% identical overall, their coiled-coil domains are only 50% identical. Therefore, one possible explanation of different functions for ARNO and GRP1 is that they 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 [59-61]. IPCEF interacts with both ARNO and GRP1 by 2-hybrid although only the interaction with ARNO was confirmed with recombinant proteins [33]. GRSP1 interacts with GRP1 and it is not known
whether this protein can also bind to ARNO [62]. It has been shown that these proteins co-localize with cytohesins and modify their ARF6 activating activity [33]. 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 additional possible explanation for the different actions of ARNO and GRP1 is the presence of a protein kinase C (PKC) phosphorylation site in the polybasic domain of ARNO that is absent in GRP1 [36]. The cytohesin polybasic domain is an intramolecular autoinhibitory domain [47]. The polybasic domain and the linker region between the Sec7 and PH domains act as a pseudosubstrate to occlude the active site. This interaction prevents the Sec7 domain from binding ARFs and inhibits ARF activation. 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 [47]. PKC activation in ARNO-expressing Hela cells produces extensive ruffling and actin rearrangement [36]. β1 integrin recycling may depend upon activation of PKC that GRP1 cannot respond to.

As the first step to define the sequence differences behind the distinct functions of ARNO and GRP1 during cell adhesion, spreading and integrin recycling, I constructed domain swapped ARNO/GRP1 fusion DNA constructs. Primarily, I constructed myc-tagged ARNO and GRP1. Then, the coiled-coil (CC) domain, PH and PB domain together or the PB domain of ARNO was swapped with GRP1 CC, PHPB or PB domains by using mutagenesis PCR reactions. GRP1 with ARNO CC, PHPB or PB domains was also constructed (Fig. 5.1).
**Figure 5.1 Constructs of domain swapped cytohesin 2/ ARNO and cytohesin 3/ GRP1**

First, Myc tagged ARNO and GRP1 were constructed. Then, ARNO with GRP1 coiled-coil, PHPB or PB domain and GRP1 with ARNO coiled-coil, PHPB or PB domain were constructed by PCR mutagenesis.
Cell Adhesions of domain swapped ARNO and GRP1

Since it was shown in chapter 3 that ARNO and GRP1 have opposing effects on cell adhesion to fibronectin, I decided to use the cell adhesion assay to determine the sequence differences causing the opposing effects on cell adhesion, spreading and migration of ARNO and GRP1. As described in Materials and Methods, myc-tagged ARNO with GRP1 CC, PHPB or PB domain was transfected into Hela cells, and the cells were plated on 5ug/ml of fibronectin for 1hr. If the relevant domain of GRP1 has been swapped into ARNO, then the cells expressing that construct will not show enhanced binding to fibronectin when compared to control cells. ARNO w/ GRP1 CC or ARNO w/ GRP1 PB expression showed significantly enhanced cell adhesion compared to the control (Fig. 5.2) suggesting that these domains do not underlie the differences between ARNO and GRP1. On other hands, cells expressing ARNO w/ GRP1 PHPB showed similar levels of cell adhesion to the control (Fig. 5.2).

Since ARNO w/ GRP1 PHPB did not show the enhanced adhesion even though ARNO w/ GRP1 PB showed enhanced adhesion, I decided to construct ARNO w/ GRP1 PH and GRP1 w/ ARNO PH hybrids for further investigation (Fig 5.3). Then, I tried the adhesion assay with ARNO w/ GRP1 PH expressing cells and found that ARNO w/ GRP1 PH expression reduced cell adhesion significantly when compared to cells’ expressing control or wild-type ARNO (Fig. 5.4). Importantly, cells expressing ARNO w/GRP1PH adhered to fibronectin similarly to cells expressing wild-type GRP1 (Fig. 5.4).
Figure 5. 2 ARNO with GRP1 PHPB expression does not enhance cell adhesion.

GRP1 PHPB domain affects ARNO expressing cell adhesion. Hela cells were transfected with myc-tagged ARNO w/ GRP1 CC, ARNO w/ GRP1 PHPB or ARNO w/ GRP1 PB. Cells were nonenzymatically harvested and allowed to bind to 5ug/ml of fibronectin for 60min. Cells were washed, fixed and stained with crystal violet. The stain was extracted into 1% Deoxycholate and OD$_{595}$ determined. Data shown are mean ± standard deviation of triplicate samples and are representative of more than 5 separate experiments. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.
Figure 5. 3 Constructs of domain swapped ARNO and GRP1

**ARNO (3G) vs. GRP1 (2G)**

The PH domain of cytohesins mediates membrane localization by binding to phosphoinositides such as PIP2 and PIP3 [26, 38]. Splice variants of cytohesin 1, cytohesin 2/ARNO and cytohesin 3/GRP1, which only differ in the number of glycine residues within the PH domain, determine PIP2 and PIP3 affinity [40, 43]. The diglycine form has a strong selectivity for PIP3, but the triglycine form has an equal affinity for PIP2 and PIP3. ARNO primarily exists as a triglycine form, but GRP1 is in a diglycine form. Since it was demonstrated...
that the PH domain of ARNO is important in cell adhesion to fibronectin (Fig. 5.4), I decided to make alternative hybrid constructs, ARNO w/ GG PH (2G ARNO) and GRP1 w/ GGG PH (3G GRP1), which only differ in glycine numbers when compared to wild-type ARNO and GRP1 (Fig. 5.3). As shown in figure 5.5, 2G ARNO expression reduced cell adhesion, and the reduced adhesion was significantly different from control and wild type ARNO (3G) adhesion. Based on all data (Fig. 5.1-5.5), I concluded that the PH domain of ARNO and GRP1 induce different effects on cell adhesion. In addition, the number of glycine residues of the PH domain is crucial to induce this phenotype.

As cells bind more tightly to fibronectin, they also spread more rapidly on the matrix. Therefore, I tried the cell spreading assay as described on Materials and Methods to see how fast cells expressing 2G ARNO and 3G GRP1 spread on fibronectin compared to cells expressing wild-type ARNO (3G) and GRP1 (2G). Consistent with the adhesion data, 2G ARNO expressing cells spread less than control cells and similar to cells expressing wild-type GRP1 (2G) (Fig. 5.6). Furthermore, 3G GRP1 expressing cells spread more than control cells and similar to cells expressing wild-type ARNO (3G) (Fig. 5.6). These data also support that the number of glycine residues in the PH domain of ARNO and GRP1, and therefore, phosphoinositide affinity is responsible for opposing effects of ARNO and GRP1 on cell adhesion and spreading.
Figure 5. 4 ARNO with GRP1 PH expression reduces cell adhesion.

ARNO w/ GRP1 PH expression reduces cell adhesion to fibronectin, while cell adhesion is enhanced by wild type ARNO expression. Myc-tagged ARNO, Flag-tagged GRP1 or Myc-tagged ARNO w/ GRP1 PH was expressed in Hela cells. Adhesion to each different concentration of fibronectin was performed as described in Fig. 5.3. Data shown are mean ± standard deviation of triplicate samples and are representative of 3 separate experiments. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.

Localization of 2G ARNO and 3G GRP1 in spreading cells

It has been shown that wild-type ARNO (3G) is localized at both the cell periphery and interior regions. However, wild-type GRP1 (2G) is exclusively located at the cell periphery in spreading cells (Fig. 4.6). In addition, wild-type ARNO (3G) is present both apical and basal
levels, however, wild-type GRP1 (2G) is mostly localized at the basal level in a Z-stack analysis (Fig. 4.7). Therefore, I hypothesized that different localization of ARNO and GRP1 is crucial to induce different effects in the cells. To support what I have shown in this chapter that the number of glycine residue is the critical factor underlying opposing effects between ARNO and GRP1, I decided to see where 2G ARNO and 3G GRP1 localize in spreading cells. MCF-7 cells co-expressing either myc-tagged 2G ARNO and flag- tagged wild-type GRP1 (2G), or myc-tagged 3G GRP1 and flag-tagged wild-type GRP1 (2G) were plated on fibronectin for 30 minutes as described in Materials and Methods. I expected that 2G ARNO would be exclusively localized at the cell periphery like wild-type GRP1 (2G), while 3G GRP1 should be located at both cell periphery and interior regions like wild-type ARNO (3G). As I expected, 2G ARNO was exclusively located at the cell periphery region which is the exact same localization as wild-type GRP1 (Fig. 5.7A). Projection along the Z-axis of the deconvolved stack also shows that 2G ARNO is restricted to the most basal level of the cell like wild type GRP1 (2G) (Fig. 5.7B). In contrast to 2G ARNO, which is exclusively located at most peripheral region similar to wild type GRP1 (2G), 3G GRP1 was localized at both peripheral and interior regions of spreading cells (Fig. 5.8 A). 3G GRP1 was located at both apical and basal levels in the cells, similar to the localization of wild-type ARNO (3G). (Fig. 4. 7B and 5. 8B).
In chapter 5, I showed that the PH domain is important for the opposing effects of ARNO and GRP1 on cell adhesion and spreading. Then, I showed that the number of glycine residues in the PH domain of ARNO and GRP1 is crucial for that phenotype. Finally, I found that the number of glycine residues also induces differential localizations of ARNO and GRP1. All data

Figure 5. 5 2G ARNO expression reduces cell adhesion to fibronectin while wild type ARNO (3G) expression enhances cell adhesion.

Myc-tagged ARNO, Flag-tagged GRP1 or Myc- tagged 2G ARNO was expressed in Hela cells. Adhesion to each different concentration of fibronectin was performed as described in Fig. 5.3. Data shown are mean ± standard deviation of triplicate samples. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.
from chapter 5 support the conclusion that ARNO and GRP1 are involved in regulation of cell adhesion and migration, and they are not redundant but have distinct functions in these processes.

Figure 5.6 Effect of 2G ARNO or 3G GRP1 expression on the spreading of MCF-7 cells

MCF-7 cells were transfected with myc-wild type ARNO (3G), myc-wild type GRP1 (2G), myc-2G ARNO or myc-3G GRP1 and spread on fibronectin as described in Materials and Methods. Spread cell surface area was measured using Slidebook 5.0 (Intelligent Imaging Innovations, Denver, CO). Spreading of each sample was normalized to the spreading of cells treated with control PCDNA3 transfection. Data shown are mean ± standard error of more than 30 cells. Data were analyzed using a T-test, asterisk indicates P<0.05; double asterisk indicates P<0.01.
Figure 5. 7 Similar localization of 2G ARNO and wild type GRP1 in spreading cells

(A) 2G ARNO and wild type GRP1 (2G) are only localized at the cell periphery region. MCF-7 cells were co-transfected with Flag-GRP1 and myc-2G ARNO, and spread on fibronectin-coated coverslips for 30 minutes. 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 the wild type GRP1 (2G) is pseudocolored green and the 2G ARNO is pseudocolored red. (B) A z-stack also showed the same localization of 2G ARNO and wild type GRP1 at only basal level. A maximum projection along the X-axis is shown. Bar, 10µm.
Figure 5.8 Differential localizations of 3G GRP1 and wild type GRP1 in spreading cells

(A) 3G GRP1 and wild type GRP1 (2G) showed differential localizations in MCF-7 cells. 3G GRP1 was localized at both cell periphery and interior regions while wild type GRP1 is only located at the cell periphery region. MCF-7 cells were cotransfected with Flag-GRP1 (2G) and myc-3G GRP1, spread on fibronectin and fixed as Fig. 5.7. 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 wild type GRP1 (2G) is pseudocolored green and 3G GRP1 is pseudocolored red. Bar, 10µm. (B) A z-stack also showed the differential localizations of 3G GRP1 and wild type GRP1. 3G GRP1 was localized at both apical and basal regions while wild type GRP1 is only located at the basal level. A maximum projection through the X-axis of the deconvolved stack in B is shown in order to visualize the location of wild type GRP1 (2G) and 3G GRP1 in the Z-dimension. Bar, 10µm.
CHAPTER 6

Discussion
The research for this thesis reveals differential effects of cytohesin 2/ARNO and cytohesin 3/GRP1 on cell adhesion and migration. First, the current research found that two similar ARF6 GEFs, ARNO and GRP1, have distinct functions in cell adhesion, spreading, and migration. ARNO enhances cell adhesion and migration, while GRP1 reduces cell adhesion and migration. Second, this study also found that only ARNO, not GRP1, is required for β1 integrin recycling. Third, ARNO and GRP1 have differential localization. ARNO localizes at both peripheral and interior regions of spreading cells while GRP1 locates only at the peripheral edges of spreading cells. Fourth, the research showed that the PH domains of ARNO and GRP1 are crucial for promoting differential effects of ARNO and GRP1 on cell adhesion and spreading. Lastly, the number of glycine residues in the PH domain of ARNO or GRP1 induces opposing effects on cell adhesion and spreading, and the localization of ARNO and GRP1 in the spreading cells.

ARF6 is a small GTPase which functions as a molecular on/off switch. GTPases cycle between active GTP- and inactive GDP- bound forms. GTPases are activated by GEFs and inactivated by GAPs [26-27]. Despite the existence of 15 ARF GEFs, mammalian cells only express 6 ARFs [28, 63]. This suggests that ARF activation is regulated by different GEFs in different signaling pathways. The 15 Sec7 family members of ARF-GEFs constitute 5 classes: GBF/BIG, Cytohesins, EFA6, BRAGs and Fbox [31]. Among them, cytohesins, EFA6 and BRAGs activate ARF6. As described, the research focuses on differential functions of cytohesins in cell adhesion and migration.
Differential functions of cytohesin 2/ARNO and cytohesin 3/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 that includes coiled-coil, sec7, PH and polybasic domains [31]. Among the cytohesin family members, ARNO and GRP1 are ubiquitously expressed and very closely related (80% identity) [40]. Previous research demonstrated that knockdown of either ARNO or GRP1 has similar effects on insulin signaling [55]. The current research demonstrates different functions of ARNO and GRP1 in cell adhesion, spreading, and migration. ARNO knockdown reduces cell adhesion, spreading, and migration, while GRP1 knockdown has opposing effects on these processes. By all accounts and investigation of previous research, this finding is the first demonstration of differential actions of ARNO and GRP1.

Differential localization of cytohesin 2/ARNO and cytohesin 3/GRP1

In addition to the different actions of cytohesin 2/ARNO and cytohesin 3/GRP1 in cell adhesion and migration, the current research indicates that these proteins have unique localizations in spreading cells. GRP1 exclusively locates 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 at these locations as cells spread. ARNO co-localizes with GRP1 in these protrusive regions and locates throughout the interior of the cell, as well. A likely conclusion is that some centrally located ARNO promotes the differential effects of ARNO and GRP1 on cell adhesion and migration.
Opposing functions of cytohesin 2/ARNO and cytohesin 3/GRP1 on cell adhesion and spreading induced by the PH domain

Although cytohesin 2/ARNO and cytohesin 3/GRP1 share 80% sequence identity, results show that ARNO and GRP1 have differential effects on cell adhesion, spreading, and migration. By constructing domain swap ARNO/GRP1 hybrids, this study identifies the sequence differences of ARNO and GRP1 responsible for the opposing effects on cell adhesion, spreading, and migration. Conducting cell adhesion and spreading assays revealed that the PH domain of cytohesins is the critical region underlying the differential effects of ARNO and GRP1 on cell adhesion and spreading. ARNO with GRP1PH expression significantly reduces cell adhesion compared to the control. Adhesion of cells expressing ARNO with GRP1PH is also significantly different from the cells expressing wild-type ARNO, but the same as the cells expressing wild-type GRP1. Another discovery is that the number of glycine residues in the PH domain is critical for these phenotypes. 2G ARNO acts similarly to wild-type GRP1 (2G), and 3G GRP1 functions like wild-type ARNO (3G) in cell adhesion and spreading. In addition, the research demonstrates that the number of glycine residues in the PH domain is an important regulator for localization of cytohesins in the cell. 2G ARNO exclusively locates at the cell peripheral edges like wild-type GRP1 (2G), while 3G GRP1 localizes at both peripheral edges and interior region of spreading cells like wild-type ARNO (3G). Based on all the data, we conclude that the PH domain of cytohesins, and the number of glycine residues in particular, is the critical determinant of cytohesin function in cell adhesion and spreading.

Previous research showed that the PH domain is involved in cytohesin recruitment to membranes. Cytohesins are recruited to the plasma membrane in response to phosphatidylinositol-3 kinase (PI3K) signaling [31]. PI3kinase metabolizes phosphatidylinositol-
4,5-diphosphate (PIP2) to produce Phosphatidylinositol 3,4,5-triphosphate (PIP3). PH domains bind to PIP2 or PIP3, and this binding mediates cytohesins’ membrane localization [26].

Cytohesin 2/ARNO and cytohesin 3/GRP1 each have two isoforms that differ only by the number of glycine residues in the PH domain. ARNO primarily exists in the triglycine form while GRP1 primarily exists in the diglycine form. Interestingly, the triglycine isoform has equal affinity for PIP2 and PIP3 while the diglycine isoform has strong preference for PIP3 [43]. The current research shows that ARNO and GRP1 have different subcellular locations while that 2G ARNO and wild-type GRP1 (2G) have similar localization. Likewise, 3G GRP1 and wild-type ARNO (3G) locate in similar regions of the cells. Furthermore, 3G GRP1 and wild-type ARNO (3G) have the same effects on cell adhesion and migration as do wild-type GRP1 (2G) and 2G ARNO. Therefore, the logical conclusion is that ARNO and GRP1 recruitment to different membrane domains by their different selectivity for PIP2 and PIP3 may be responsible for their differential effects on cell adhesion, spreading, and migration.

**β1 integrin recycling pathway**

Cell migration is regulated by trafficking of integrins which are continuously recycled by endocytosis and reinsertion into the plasma membrane [64-65]. Integrin is redistributed from the trailing edge to the leading edge of migrating cells [66]. ARF6 is known to regulate both β1 integrin endocytosis and recycling [20-21].

The itinerary taken by ARF6-dependant cargo has been elucidated. These cargo proteins, such as β1 integrin, are internalized by clathrin-independent endocytosis (CIE). They then enter the early endosome where they meet up with cargo that was internalized in a clathrin-dependent manner. Recycling cargo then travels to the Rab11 positive endosomal recycling compartment.
ARF6 dependent cargo is then returned to the plasma membrane via tubular recycling endosomes that align along microtubules. Both the internalization and recycling steps of this pathway require ARF6 activity [39].

Previous research demonstrated that Brag2 localizes to the plasma membrane and activates ARF6 to regulate β1 integrin endocytosis [23]. It also has been demonstrated that ACAP1, a GAP for ARF6, is involved in recycling of β1 integrin [20]. However, the ARF6 GEF involved in β1 integrin recycling remained unknown. According to previous investigation, cytohesin 2/ARNO stimulates epithelial cell migration, and cytohesin 3/GRP1 induces actin cytoskeleton rearrangement [38, 67]. Recycling of adhesion proteins is one of the mechanisms for regulating cell migration. Therefore, I tested ARNO and GRP1 as promising candidates and found that only ARNO is required for β1 integrin recycling. This result likely explains the differential effects of ARNO and GRP1 on adhesion, spreading, and migration.

**Integrin Traffic**

Integrins enter cells via an ARF6–associated CIE mechanism [39]. Brag2 activates ARF6 to regulate internalization of β1 integrin [23]. Rab5 is involved in this process by serving as a scaffold to organize and coordinate the trafficking [39]. Once internalized, β1 integrin transfers to the early endosome, which receives materials internalized via both clathrin-dependent endocytosis (CDE) and CIE mechanisms. At the early endosome, β1 integrin meets with clathrin cargoes, which either degrade or recycle back to the plasma membrane. β1 integrin can move from the early endosome to endocytic recycling compartment (ERC) with the assistance of Rab21 and FIP2, -3 and -5, Rab11 effectors. Then, Rab11, Rab22 and Rab8
regulate β1 integrin traffic from ERC to the recycling endosome [39]. Under serum-starved conditions, β1 integrin is accumulated at the recycling endosome where ARF6, Rab 11 and ACAP1 localize, while ARNO localizes primarily at the cytoplasm [20-21, 68] (Fig.6.1A). However, serum stimulation recruits ARNO to the plasma membrane and onto endocytic structures [68] (Fig. 6.1. B).

PIP2 is produced by phosphatidylinositol phosphate kinases (PIPKs), specifically phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) [68-69]. PIP2 localizes at both the plasma membrane and the endosome. PIP2 synthesis has been implicated in the regulation of membrane recycling between endosomal compartments and the plasma membrane [68-69]. In contrast to PIP2, PIP3 is detected at the plasma membrane only in stimulated cells [70]. PIP3 synthesis at the plasma membrane appears after the stimulation of cells by growth factors [46]. ARNO is recruited to membranes by binding its PH domain to PIP2 and PIP3 [27, 31, 46]. Binding of the PH domain of ARNO to PIP2 and PIP3 concentrates ARNO onto the membranes, thereby increasing the level of active ARF6 [28].

Stimulation of cells with serum will induce generation of PIP2 and PIP3 and the relocalization of ARNO and GRP1 (Fig. 6.1B). PIP2 is synthesized and localized at both the plasma membrane and endocytic compartments while PIP3 is only synthesized at the plasma membrane upon serum stimulation. ARNO is recruited to the plasma membrane and the endosome via binding to both PIP2 and PIP3 [27, 31, 46]. We hypothesize that ARNO recruitment at the recycling endosome regulates β1 integrin recycling by activating ARF6. ARF6, Rab11, Cdc42 and cortical actin promotes the recycling of β1 integrin by breaking up tubules into vesicles that then fuse with the plasma membrane (Fig. 6.1B) [39].
Figure 6.1 Model for β1 integrin recycling (continued)

(A) Under serum-starved condition, ARNO and GRP1 are cytosolic, and β1 integrin is accumulated at the recycling endosome (RE) where ACAP1 and Rab11 localize.
Figure 6.1 Model for β1 integrin recycling

(B) Serum stimulation induces generation and localization of PIP2 at the plasma membrane and the endosome, and PIP3 at the plasma membrane. ARNO will be recruited to both the plasma membrane and the recycling endosome by binding to PIP2 and PIP3, while GRP1 will be only recruited to the plasma membrane by binding to PIP3. ARNO, at the recycling endosome, will activate ARF6 to regulate β1 integrin recycling with ACAP1, Rab11, and cortical actin. EE: Early endosome ERC: Endocytic Recycling Compartment
Counteracting function of GRP1

In contrast to ARNO, GRP1 is not involved in β1 integrin recycling. GRP1 will localize only to the plasma membrane by binding to PIP3 upon serum stimulation. Since cytohesins are closely related proteins, ARNO and GRP1 may share many up- and downstream signaling molecules (Fig. 6.2). Several proteins associated with the cellular functions of ARF6 have been studied. Scaffolding proteins are upstream molecules that coordinate cytohesins in ARF6 signal transduction pathways. Scaffolding proteins such as GRASP and IPCEF regulate ARF6 activity by binding to the coiled-coil domain of ARNO [50]. Cytohesins activate ARF6 and thereby turn on many downstream signal transduction pathways [71]. Rac, PIP5K and PLD are well studied downstream molecules in ARF6 signaling pathways (Fig. 6.2) [72]. GRP1 expression in cells can affect all of the signaling pathways shown in Fig.6.2. GRP1 expression may reduce cell adhesion and spreading by sequestering these common up- or downstream components and thereby perturbing formation of a functional complex of ARNO and ARF6 with effectors (Fig.6.3). To distinguish whether GRP1 affects up- or downstream signaling in the differential functions of ARNO and GRP1, catalytically inactive GRP1, E161K, could be utilized. GRP1E161K will sequester upstream components, but will not activate ARF6, and therefore will not sequester downstream components. Therefore, the effect of GRP1E161K over-expression on cell adhesion will directly determine whether the sequestered component is upstream or downstream. Obviously, further investigation for the counteracting function of GRP1 on ARNO is required.

Although 15 ARF-GEFs exist in mammalian cells, their functions remain partially undefined. The current research found that cytohesin 2/ARNO and cytohesin 3/GRP1 have distinct functions in cell adhesion and migration. The number of glycine residues in the PH domain of ARNO and GRP1 is crucial to induce the differential function and the differential
localization of ARNO and GRP1. This study also found that only ARNO, not GRP1, regulates β1 integrin recycling. Binding specificity of ARNO and GRP1 to PIP2 and PIP3 may differentiate localization and function of ARNO and GRP1. This proposed model provides a possible mechanism how cytohesin 2/ARNO regulates β1 recycling, and thereby ARF6 controls cell migration. This is the first demonstration of unique roles for cytohesin 2/ARNO and cytohesin 3/GRP1, and the mechanism underlying the differing actions of these proteins.

Figure 6.2 ARF6 signaling pathways

ARNO and GRP1 may share many up- and downstream signaling molecules.
Figure 6.3 Model for counteracting function of GRP1 (continued)

(A) Serum stimulation induces β1 integrin recycling described in Fig. 6.1.
Figure 6.3 Model for counteracting function of GRP1

(B) GRP1 expression may inhibit β1 integrin recycling by sequestering necessary common signaling molecules away from ARNO.
CHAPTER 7
Future directions
Rescue of integrin recycling

In chapter 5, I showed that the number of glycine residues, either two glycines (2G) or three glycines (3G), in the PH domain determines the functions of cytohesin in cell adhesion and spreading. 3G GRP1 and wild type ARNO (3G) expression enhances cell adhesion and spreading, while 2G ARNO and wild type GRP1 (2G) expression reduces cell adhesion and spreading. It was also shown that the number of glycine residues in the PH domain affects cytohesin localization. 3G GRP1 and wild type ARNO (3G) were localized at both cell protrusive and interior regions, while 2G ARNO and wild type GRP1 (2G) were only located at the cell periphery. In chapter 4, I demonstrated that ARNO, not GRP1, regulates β1 integrin recycling. Due to the differential localizations of ARNO and GRP1, I hypothesized that centrally located ARNO may promote β1 integrin recycling from recycling endosomes to plasma membrane. Since only ARNO, not GRP1, is involved in β1 integrin recycling, and 3G GRP1 acts and localizes as ARNO, I decided to test whether 3G GRP1 expression can rescue impaired β1 integrin recycling in the cells with reduced ARNO expression.

Experimental approach

To test whether 3G ARNO can reverse the defects in the cells with reduced ARNO expression, I will try the β1 integrin recycling assay described in chapter 2. Two days before the assay, siRNA targeting ARNO will be transfected into MCF-7 cells by using Neon Transfection System (Invitrogen). The day before the assay, control plasmid or 3G GRP1 will be transfected
into the cells expressing reduced ARNO. Then, I will try the β1 integrin recycling assay to see whether 3G GRP1 can reverse impaired β1 integrin recycling caused by reduced ARNO expression. The cells with control transfection will still show impaired β1 integrin recycling. However, if the number of glycine residues in the PH domain is an important regulatory factor for β1 integrin recycling mechanism, 3G GRP1 expression will rescue β1 integrin recycling defects caused by reduced ARNO expression.
Investigation of co-localization of cytohesin 2/ARNO and cytohesin 3/GRP1 with PIP2 and PIP3

Cytohesin 1, cytohesin 2/ARNO and cytohesin 3/GRP1 have two splice variants corresponding to the number of glycine residues, either 2G or 3G, in the PH domain. The triglycine splice form of cytohesin has been shown to have an equal affinity for PIP2 and PIP3, while the diglycine variants strongly prefer PIP3 binding. Cytohesin 1 and cytohesin 2/ ARNO are primarily in triglycine forms, while cytohesin 3/ GRP1 is usually in the diglycine form. It was shown in this thesis that 3G GRP1 functions similar to 3G ARNO, and 2G ARNO also functions similar to 2G GRP1 in cell adhesion and spreading. In light of these findings, I should test whether 3G GRP1 and ARNO co-localize with PIP2 and PIP3, and 2G ARNO and GRP1 co-localize with PIP3 only. It was shown that 3G GRP1 and ARNO are localized at the cell peripheral and interior regions while 2G ARNO and GRP1 are only located at the cell peripheral region. These subcellular locations are consistent with the 2G isoforms binding to PIP3, which is primarily at the plasma membrane, and the 3G isoforms binding to both PIP2 and PIP3. Directly demonstrating that the 2G isoforms localize with PIP3 while the 3G isoforms also localize with PIP2 would support the importance of differential localizations of ARNO and GRP1 in cytohesin functions. In addition, these results would broaden the link between cytohesin functions and lipid signal transduction.
**Experimental approach**

PtdIns (4,5)P2 (PIP2) has been shown to be localized at sites of actin polymerization and membrane ruffling and thus regulate cell shape and motility. Since PH\textsubscript{PLC\textsubscript{δ1}} interacts with the inositol head group of PtdIns (4,5)P2, the PH\textsubscript{PLC\textsubscript{δ1}}-GFP probe has been used to detect PIP2 [70]. Moderate levels of PH\textsubscript{PLC\textsubscript{δ1}} expression has been shown not to significantly alter cell physiology. Therefore, PH\textsubscript{PLC\textsubscript{δ1}}-GFP will be used as an indicator of PIP2. To test whether PIP2 and 3G GRP1 colocalize in spreading cells, PH\textsubscript{PLC\textsubscript{δ1}}-GFP and 3G GRP1 or 2G ARNO will be coexpressed in MCF-7, and the spreading assay will be conducted. Then, the cells will be fixed and stained as described in chapter 2. For the control experiment, PH\textsubscript{PLC\textsubscript{δ1}}-GFP and wild-type ARNO or wild-type GRP1 will be coexpressed in the cells to conduct the same experiment. I expect that 3G GRP1 will colocalize with PIP2 while 2G ARNO will not show colocalization with PIP2 based on the data we shown in chapter 4 and 5.

PtdIns (3,4,5)P3 (PIP3) is found at the plasma membrane only when cells are stimulated while PIP2 is permanently located at the plasma membrane. PIP3 synthesis has been detected by PH\textsubscript{Btk}-GFP after stimulation with EGF or Platelet derived growth factor (PDGF) [70]. Therefore, PH\textsubscript{Btk}-GFP will be used as an indicator of PIP3 in this experiment. To test whether 2G ARNO or 3G GRP1 colocalizes with PIP3 in spreading cells, PH\textsubscript{Btk}-GFP and 2G ARNO or 3G GRP1 will be coexpressed in MCF-7, and the cells will be stimulated by EGF before conducting the spreading assay. I expect that 2G ARNO will show the same localization with PIP3 as 3G GRP1 will partially share the localization with PIP3.
**E-cadherin recycling study**

My thesis project mainly focused on the study of differential functions of cytohesins on cell adhesion and migration. As part of the project, cytohesin involvement in β1 integrin recycling was tested, and I demonstrated that only ARNO, not GRP1, regulates the recycling. It has been shown that ARF6 regulates not only endocytosis and recycling of β1 integrin but also endocytosis of E-cadherin. E-cadherin is a homodimeric adherens junction protein for cell-cell adhesion. Like other junction proteins, cell surface expression of E-cadherin is determined by the balance between transport of the protein to the plasma membrane and endocytosis of the protein into the cell [73]. This process has been suggested to regulate cell movement as well as cell junction formation. ARF6 is known to regulate endocytosis of E-cadherin from sites of cell-cell contact to the early endosome [49]. However, E-cadherin recycling mechanism is still unclear.

Epithelial to Mesenchymal Transition (EMT) is a developmental program whereby epithelial cells lose adhesions and acquire motility [8]. Epithelial cancers also acquire a phenotype similar to EMT when they metastasize [74]. As described in chapter 1, E-cadherin is important regulator for cell migration. E-cadherin is down regulated during EMT and metastasis, and E-cadherin expression can prevent metastasis in metatic cancer cells [9, 13]. Since ARF6 regulates endocytosis and recycling of various cell surface proteins, I will test whether ARF6 regulates E-cadherin recycling. In addition, involvement of cytohesins in E-cadherin recycling pathway will be also tested by the calcium switch assay.
Experimental approach

The major experimental approach for this trial is a calcium-switch assay. A calcium-switch assay is a broadly used technique for E-cadherin studies. Because E-cadherin mediated adhesion is calcium dependent, the cells open the junctions if calcium is depleted. Briefly, the cells are calcium depleted to internalize E-cadherin, and then calcium is restored to recycle E-cadherin back to the cell surface in calcium switch assay (Fig. 7.1). A surface biotin labeling mediated E-cadherin recycling assay is the same as the surface antibody binding techniques described in chapter 2 except biotin binds all surface proteins rather than one specific protein. First, junction formation in ARNO or GRP1 knockdown cells will be observed by a calcium-switch assay to determine if these proteins are involved in E-cadherin recycling. It was in this report that cytohesins are required for E-cadherin recycling (Fig. 7.2). It is known that only ARNO and GRP1 are ubiquitously expressed in the cell. Since ARF6 regulates cell migration, E-cadherin recycling requires cytohesins, and E-cadherin recycling is an important part of cell migration, I hypothesize that either ARNO or GRP1 may regulate the recycling of E-cadherin.

Control and ARNO or GRP1 will be knocked down in MCF-7 by siRNA. After knockdown of the individual proteins, the cells will be incubated in low calcium medium to open junctions, and then the medium will be switched to normal calcium medium at 37°C. After 0 and 3 hours of incubation in normal medium, the samples will be fixed and stained with fluorescent anti-mouse secondary antibody for E-cadherin to observe junction formation. After 3 hours incubation in normal calcium containing medium, the cell will reform junctions (Fig. 7.1 B).
Figure 7.1 E-cadherin Recycling was detected by the surface biotin labeling technique and the Calcium-Switch Assay.

(A) The cell surface was labeled with biotin, and labeled surface E-cadherin was accumulated in the intracellular pool by incubating the cells at 18°C. Remaining labeled E-cadherin on the cell surface was stripped, and then E-cadherin was released to the plasma membrane by incubating in 37°C. 0, 5 and 15min after recycling initiation, recycled biotin was removed again from the cell surface. Finally, E-cadherin was detected by Western blot after Immunoprecipitation of internal biotin with streptavidin beads. (B) After calcium depletion, calcium was restored for 0 and 3hrs. Cells were stained for E-cadherin.

For a surface binding of biotin, Sulfo-NHSS biotin (Pierce) will be applied to the MCF-7 cells at 4°C, and then the cells will be warmed to 18°C to accumulate E-cadherin in the intracellular pool. Residual biotin remaining on the cell surface will be removed with stripping buffer (50 mM MesNa, 50 mM Tris (pH 8.6), 100 mM NaCl) on ice, and then the samples will be incubated at 37°C to initiate E-cadherin recycling. After 0 and 15 minutes of recycling initiation, the recycled biotin will be stripped, and the samples will be lysed and incubated with streptavidin beads to isolate internal biotin-bound E-cadherin. Finally, the samples will be analyzed by Western blot for E-cadherin. After 15 minutes of recycling initiation, most of E-cadherin was recycled back to the cell surface (Fig. 7.1 A).
Reexpression experiments will be used to confirm that all results made during knockdown experiments are due to knocking down of the particular proteins and not due to off-target (non-specific effects). The knockdown effect will be also confirmed by using different siRNAs (more than 1 per each protein) to knock down the proteins. In addition, overexpression of ARNO or GRP1 will be performed to see if that accelerates recycling.

Figure 7.2 E-cadherin recycling requires cytohesins.

SecinH3 treated cells were calcium restored for 3 hours after calcium depleting mediated junction opening
REFERENCES


67. Clodi, M., et al., *Effects of General Receptor for Phosphoinositides 1 on Insulin and Insulin-Like Growth Factor I-Induced Cytoskeletal Rearrangement, Glucose*


Appendix A: siRNA knockdown efficiency

Figure A-1 Transfection of siRNA reduces expression of cytohesin 2/ARNO and cytohesin 3/Grp1.

Hela, MCF-7, and MDCK cells were transfected with the indicated siRNAs as described in Experimental Procedures. Total RNA was isolated and RT-PCR performed with 0.5 µg of RNA as template as described in Experimental Procedures.
APPENDIX B: Source of contents


• Seung Ja Oh and Loraine C. Santy, “Opposite regulation of similar molecules, cytohesin 2 and 3 by Pleckstrin Homology (PH) domain” (In preparation)
VITA

SEUNG JA OH

EDUCATION

- Ph. D. Biochemistry, Microbiology and Molecular Biology (2006-2011)
  The Pennsylvania State University, USA

- B.S. Biochemistry and Molecular Biology (2003-2005)
  The Pennsylvania State University, USA

AWARDS AND HONORS


- Braucher Fellowship Award, May. 2008, The Pennsylvania State University

- Braddock Fellowship Award, Sep. 2006, The Pennsylvania State University

PUBLICATIONS

- Seung Ja Oh and Loraine C. Santy, “Opposite regulation of similar molecules, cytohesin 2 and 3 by the Pleckstrin Homology (PH) domain” (In preparation)
