THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

INVOLVEMENT OF THE INTERACTIONS OF THE COILED COILED DOMAIN AND Δ-COILS ARNO IN THE LOCALIZATION OF ARNO/CYTOHESIN-2

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Abstract

Epithelial cells are typically stationary in an organism, but become motile during wound healing, tissue morphogenesis, and metastasis of epithelial tumors. Previous research has supported the role of ARF6 in epithelial migration activity. As a GTPase, ARF6 is regulated by a guanine nucleotide exchange factor known as ARNO or cytohesin-2. When ARNO is recruited to the membrane from the cytosol, it is able to activate ARF6. This research demonstrates the altered localization of ARNO with the use of a recombinant ARNO, known as Δ-coils ARNO, in which the coiled coil domain of ARNO is removed. This evidences the integral role of the coiled coil domain of ARNO in the localization of ARNO to the membrane. Furthermore, upon incubation of Δ-coils ARNO with the coiled coil domain of ARNO, an interaction was demonstrated between the coiled coil domain and the Δ-coils ARNO. This speculated interaction would block the PH domain, a membrane binding domain of ARNO, resulting in the retention of ARNO in the cytosol. This proposed mechanism is predicted to be carried out through the activity of scaffold proteins, which is also tested in this study.

Acknowledgements

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Table of Contents

Introduction

Epithelial Cell Migration

Epithelial cells form sheets by adhering to adjacent epithelial cells through junctional complexes. These complexes provide mechanical strength and prevent small molecules from passing through the barrier. Customarily, the epithelial cells found in tissues are stationary, however, during wound healing, embryonic development and the metastasis of tumors, epithelial cells may become motile (Gumbiner, 1996). Cell migration has also been shown to be initiated by several growth factors including the hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (Hay, 2005). Treatment of cells with HGF in vitro results in many epithelial cell lines to become motile and scatter (Montesano et al., 1991). For this reason, expression of HGF increases in response to cell damage (Neuss et al., 2004) as well as during tumorigenesis (van der Voort et al., 2000).

The process of cell migration is essential for many aspects of developmental processes. It requires several spatially and temporally coordinated changes in cytoskeletons and cellular membranes, utilizing dynamic structures such as filopodia, lamellipodia and actin stress fibers (Tomohiro et al., 2010). Intracellular signaling creates a cell polarity, which allows a cell to move productively in one direction. As movement is initiated, actin polymers are reorganized to extend a protrusion. If the actin forms a dendritic network, broad lamellipodia form. Conversely, if actin bundles align parallel to one another, spike-like filopodia can form. These two types of protrusions then adhere to the extracellular matrix, allowing the cell to pull itself forward. Once it has reabsorbed a protrusion, the adhesions are disassembled at the rear (Ridley et al., 2003).

ARF6

Previous research has supported the role of the ADP-ribosylation factor (ARF) family of small GTPases in the cortical actin rearrangements (Radhakrishna et al., 1996). ARFs are a family of Ras-related GTP-binding proteins that function in the regulation of membrane trafficking and structure. The six mammalian ARF proteins are expressed ubiquitously. The ARF family proteins are classified into three classes. Class I and class II contains ARF1 to ARF3 and ARF4 and ARF5, respectively. Only one ARF comprises class III and is known as ARF6 (Chavrier and Goud, 1999). The separate classification of ARF6 from the rest of the ARF family, suggests the unique nature of the protein. Literature has shown that endogenous expressed ARF6 is localized to the apical plasma membrane (Altschuler et al., 1999). The association of ARF6 with the plasma membrane only occurs when ARF6 is in the active form (Gaschet and Hsu, 1999). As a small GTPase, ARF6 cycles between an active GTP-bound form and an inactive GDP-bound form (Gaschet and Hsu, 1999). Like other GTPases, this action is regulated by a class of proteins known as guanine nucleotide exchange factors (GEFs), which promotes the binding of GTP and activates the GTPase.

Regulation of ARF6 by ARNO

ARNO, also known as cytohesin-2, is a member of one subfamily of ARF-GEFs. Within the protein, there are four main domains, which can be observed from Figure 1. The sec7 domain is the catalytic ARF-GEF domain. The pleckstrin homology (PH) mediates binding to membrane surfaces and interacts with other proteins. The polybasic domain also mediates binding to membrane surfaces, but can act as an intramolecular

inhibitory domain (DiNitto et al., 2007). The coiled coil domain promotes dimerization and interacts with a number of other proteins (Casanova, 2007). Specifically, it has been shown to interact with several small scaffold proteins. These include GRASP/Tamalin (Nevrivy et al., 2000) and Pip3-E/IPCEF (Venkateswarlu, 2003).

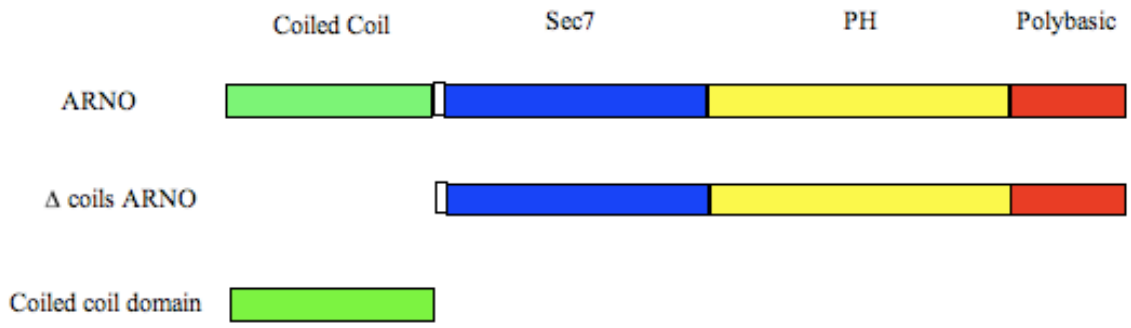

Figure 1: Domains present in wild-type ARNO, Δ coils construct and coiled coil domain. The wild-type ARNO contains all four domains. The Δ-coils ARNO construct is lacking the coiled coil domain. The coiled coil domain has been isolated in the third construct.

ARNO has been shown to have specificity for ARF6 in MDCK cells. Cells expressing wild-type ARNO displayed levels of enhanced GTP-ARF6, supporting the role of ARNO in activating ARF6 (Santy and Casanova, 2001). This activation occurs on the membrane. Previous research has shown that endogenously expressed ARNO localizes to the apical plasma membrane, colocalizing with ARF6 in MDCK cells (Shmuel et al., 2006). Furthermore, no endogenous or exogenous expressed ARNO was detected in a perinuclear or supranuclear cytoplasmic location (Shmuel et al., 2006). The activation of ARF6 by ARNO on the membrane results in a migratory phenotype (Santy and Casanova, 2001). MDCK cells typically form compact islands of cells when grown at

subconfluent density. After expressing ARNO in these cells, it was observed that ARNO-expressing cells on the edges of the island form lamellae and initiate motility (Santy and Casanova, 2001).

Rac1 activation

Rac activation is required for the cell migration activities. Rac is part of the Rho family of GTPases (Ridley, 2001). In cells, these GTPases are involved directly in the reorganization of the actin cytoskeleton (Sit and Manser, 2011). In MDCK cells, micronjection of negative Rac1 constructs results in the inhibition of the formation of the lamellipodia responsible for the migration of cells into a wound to begin wound healing (Fenteany et al., 2000). The large lamellipodia induced by Rac1 required for the wound healing process are similar to the lamellipodia induced by ARNO (Santy and Casanova, 2001). Studies attempting to link ARNO expression to Rac activation have shown support for the downstream activation of Rac1 from ARNO expression. Expression of ARNO in MDCK cells led to robust activation of Rac1, formation of the lamellipodia associated with Rac activation and ultimately epithelial motility (Santy and Casanova, 2001).

ARNO activates Rac1 through a Rac GEF complex consisting of DOCK180 and Elmo. DOCK180 is a nonconvential Rac GEF that functions in numerous migration events, including dorsal closure in *Drosphilia* (Nolan et al., 1998) and gonadal distal tip cell migration in *C. elegans* (Wu and Horvitz, 1998). Unlike other Rac GEFs, DOCK180 lacks the DH domain responsible for GEF activity. Instead, the DOCKER domain has been found to be the catalytic region of DOCK180 (Santy et al., 2005). DOCK180 also

lacks the PH domain typically found adjacent to the DH domain in most Rac GEFs. The role of this missing domain is filled by the PH domain in the second protein, Elmo (Lu et al., 2004). For this reason, DOCK180 and Elmo must form a complex in order to have active GEF function on Rac1. This occurs through the DOCK180 binding domain of Elmo, forming the DOCK180/Elmo complex (Lu et al., 2004). The DOCK180/Elmo complex has been demonstrated to mediate the activation of Rac1 downstream of ARNO and ARF6 (Santy et al., 2005).

The exact mechanism by which the DOCK180/Elmo complex mediates Rac activation is unknown. One proposed mechanism suggests that the activation of ARF6 results in ARF6 recruiting endosomal Rac1 to the DOCK180/Elmo complex located at the plasma membrane (Santy et al., 2005). Another mechanism suggests the involvement of RhoG, another member of the Rho family of GTPases. RhoG has been shown to have binding activity with Elmo and could therefore recruit the Dock180/Elmo complex to the membrane (Santy et al., 2005).

ARNO localization is regulated by IPCEF1

IPCEF1 is one of the scaffold proteins that promotes the recruitment of ARNO to the plasma membrane, where it can activate ARF6 (Venkateswarlu, 2003; Esteban et al., 2006). This protein can therefore control the subcellular location of ARNO. Knockdown of IPCEF1 in MDCK cells results in the inability of ARNO to bind to DOCK180 (White et al., 2010). This would inhibit the further activation of Rac by the DOCK180/Elmo complex through ARNO and thus, the migration of the epithelial cells. IPCEF1 is able to localize to the membrane through binding of its PH domain to phosphoinsinotides

(PtdIns), membrane phospholipids located in the membrane (Venkateswarlu, 2003). The protein promotes the co-localization of ARNO by interacting with ARNO in its coiled coil domain (Venkateswarlu, 2003). Previous studies have demonstrated this mechanism through observations of IPCEF1 localizing to the cytosol and interacting with ARNO in cells that were not stimulated with EGF, a growth factor that promotes epithelial migration. Once the cells were stimulated with EGF, IPCEF1 relocated to the membrane with ARNO (Venkateswarlu, 2003). Furthermore knockdown of IPCEF impairs activation of ARF6 by HGF and HGF-stimulated migration (Attar et al., 2012). This supports the IPCEF1's role as a scaffold protein for ARNO.

Experimental Goal and Design

Previous studies have highlighted the coiled coil domain of ARNO as a main contributing factor in the protein's regulatory functions. Specifically, in order for ARNO to carry out activation of ARF6, a protein implicated in epithelial migration, ARNO must localize to the membrane. Deletion of the coiled coil domain results in unspecific localization of ARNO, supporting the role of the coiled coil domain in the localization of ARNO (Shmuel et al., 2006). The proposed mechanism by which the coiled coil domain carries out this localization is through interaction with a scaffold protein (Shmuel et al., 2006). This data suggested that in order for ARNO to localize to the membrane, the coiled coil domain must be accessible. Paradoxically this study found that ARNO lacking the coiled coil domain was more prevalent on membranes than is the full-length protein, which contained the coiled coil domain (Shmuel et al., 2006). Both the full-length ARNO and the Δ-coils ARNO contain the membrane binding PH and PB domains. Therefore

this paper hypothesizes that the coiled coil domain is inhibiting the interaction of these domains with the membrane surface. Furthermore, this paper hypothesizes that binding of scaffold proteins to the coiled coil domain relieves this inhibition. Thus, in this paper, the possible inhibition of the coiled-coil domain to retain ARNO in the cytosol is investigated. In particular, this paper attempts to demonstrate a possible interaction between the coiled coil domain and another domain within the protein. Revealing this interaction will provide insight into possible regulation techniques of the many epithelial cell migration activities controlled by the downstream effects of ARNO, including wound healing, embryonic development and the metastasis of tumors.

To assess the interaction of the coiled coil domain with the remaining protein, a construct, which can be seen in Figure 1, lacking the coiled coil domain was created. This Δ-coils ARNO also contained a myc tag and was used to infect MDCK cells. The coiled coil domain fused to GST was grown up in *E. Coli* and bound to glutathione beads. The Δ-coils ARNO was allowed to interact *in vitro* with the GST fused coiled coil domain and glutathione beads. The product of each was centrifuged to remove all soluble factors that did not bind to the GST fused coiled coil domain and the GST beads. The proteins that remained bound to the beads were then Western blotted with an α myc antibody to detect for the Δ-coils ARNO. Western blotting was used to assess the presence of an interaction between the coiled coil domain and Δ-coils ARNO.

The described approach was used to test for the ability of scaffold proteins to disrupt the hypothesized interaction of the coiled coil domain with the remaining regions of ARNO. Specifically, the MDCK cells were infected with myc-tagged Δ-coils ARNO or myc-tagged Δ-coils ARNO and HA-tagged IPCEF1 construct. The lysates from each of these

infections were allowed to interact *in vitro* with GST-coiled coil and GST. ARNO was allowed to interact *in vitro* with the GST fused coiled coil domain and GST. The product of each was centrifuged to remove all soluble factors that did not bind to the GST fused coiled coil domain and the GST beads. The results were then visualized with Western blotting using mouse α myc antibody to detect Δ-coils ARNO and mouse α HA antibody to detect IPCEF1.

Materials and Methods

Cells

Mammalian Cells

The T23 line of MDCK cells were maintained in DMEM supplemented with 10% FBS and 1% PSF. All cell lines were cultured at 37ºC in a 5% CO2 water-jacketed incubator.

Bacterial Cells

The *E. coli* strains used for the transformation and amplification of DNA constructs were XL1-Blue and DH5α

Antibodies

Primary Antibodies

Mouse α-myc 9E10 and mouse α-HA were purchased from Covance. Mouse α-actin and mouse α-E-cadherin were obtained from BD Biosciences.

Secondary Antibody

Goat anti-mouse horseradish peroxidase was purchased from Invitrogen.

DNA Constructs

Myc-tagged full-length ARNO was produced by amplifying native ARNO cDNA by PCR using a non-coding primer that included the c-*myc* epitope sequence as described

previously (Frank et al., 1998). The myc-tagged Δ-coils ARNO was produced by amplifying the region of ARNO encoding amino acids 56-400 using a non-coding primer that included the c-*myc* epitope sequence as described previously (White et al., 2010). An IPCEF1 cDNA was used to produce the HA-tagged IPCEF1 by amplifying the sequence with PCR using a primer containing the sequence for an HA tag as previously described (White et al., 2010). Lastly, a coiled coil domain of ARNO construct was produced by amplifying the region of ARNO encoding amino acids 1-67.

Construct Expression

E. coli *Transformation and Expression*

Constructs were added to competent DH5α cells thawed on ice and incubated for 30 minutes. The cells were then heat shocked for 90 seconds in 42° Celsius. After 2 minutes on ice, the cells were incubated at 37° Celsius for 45 minutes with SOC media. The SOC solution was then plated on LB-ampicillin plates and incubated overnight at 37° Celsius. A culture was taken from the plate and inoculated into a culture of BL21 cells with LB and ampicillin to grow overnight at 37° Celsius on a shaker. The culture was backdiluted 1:25 the next day into another culture of LB and ampicillin and grown up to an OD600 of 0.4 to 0.6. The culture was then induced with 1 mM IPTG and incubated at 37° Celsius on a shaker for 4 hours. This culture was then pelleted and stored at -80° Celsius until use.

Recombinant Adenovirus Production and Infection

A recombinant adenovirus system was used to express many of the constructs utilized in this study. In this system, a tetracycline regulated promoter followed by the constructs attempting to be expressed replaced the viral genes in an adenovirus using Cre-lox recombination (Hardy et al., 1997). The virus was then added to MDCK cells in serum-free media (DMEM supplemented 1% PSF), and incubated for 3.5 hours.

Membrane Fractionation

MDCK cells were infected with recombinant adenoviruses expressing either the full-length ARNO or the Δ-coils ARNO for 3.5 hours. The cells were then scraped off in 250 mM sucrose, 3 mM imidizole, 1 mM EDTA, 1 mM DTT and 1 mM protease inhibitors. The cells were then broken by passing them through a 22-gauge needle 8 times. Unlysed cells and nuclei were removed by spinning the cell lysate 2 times at 10,000 rpm for 10 minutes at 4° Celsius. The membranes were then pelleted by spinning the remaining supernatant at 50,000 rpm for 1 hour at 4° Celsius in a TLA 100.3 rotor. The supernatant from this spin was preserved to produce the cytosolic fraction.

Assay for assessing protein-protein interactions between coiled coil domain

From E. coli

The bacterial pellet stored at -80° Celsius was resuspended in bacterial lysis buffer (50 mM Tris pH 8.0, 20% sucrose, 10% glycerol, 20 mM DTT and 1 mM protease inhibitors) at 4° Celsius. The cells were then sonicated to break them open. The unsolublized material was pelleted out by spinning the cell lysate at 13,000 rpm for 20

mins at 4° Celsius. The remaining supernatant contained either GST alone, GST-Δ-coils, or the GST-coiled coil domain, depending on the corresponding transformation. The GST-Δ-coils ARNO and the GST-coiled coil domain were added to glutathione beads and incubated to facilitate binding. The GST tag was then cleaved off of the Δ-coils ARNO using thrombin in PBS at room temperature. Next the Δ-coils ARNO solution was centrifuged to remove the beads and the supernatant was preserved. Half of this solution was incubated with GST beads for 1-2 hours at 4° Celsius and the other half was incubated with GST-coiled coil beads for 1-2 hours at 4° Celsius. The products of each were washed with PBS 4 times and all remaining liquid was removed, leaving just what is retained on the GST or GST-coiled coil beads. The beads were eluted in SDS-PAGE buffer and run on a gel.

From MDCK cells

MDCK cells were infected with recombinant adenovirus expressing Δ-coils ARNO or recombinant adenovirus expressing Δ-coils ARNO and recombinant adenovirus expressing IPCEF1 for 3.5 hours. The cells were then lysed in Rac pulldown buffer (50 mM Tris HCl, pH 7.4, 12 mM $MgCl_2$, 10% glycerol, 100 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM sodium orthonadate, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 100 μg/ml AEBSF). From this lysate, the unsolubilized material was spun out at 14,000 rpm for 10 minutes at 4° Celsius. Half of the remaining supernatant, containing either myc-tagged Δ-coils ARNO or the myc-tagged Δ-coils ARNO and HA-tagged IPCEF1, was incubated with GST beads and the other half was incubated with GST-coiled coil for 1-2 hours at 4° Celsius. The incubation products were washed in Rac

pulldown buffer 6 times and all of the remaining liquid was removed to keep only what was retained on the GST or GST-coiled coil beads. The beads were eluted in SDS-PAGE buffer and run on a gel.

Western Blotting

Protein samples were dissolved in SDS, run on a 10% SDS gel and then transferred to a nitrocellulose membrane (Whatman®). Blocking was performed using 5% dry milk in 1X PBS-T solution for one hour at room temperature on a rocking platform. Primary antibody incubation was performed at room temperature for one hour with 1:1,000 primary antibody diluted in PBS-T. Membranes were washed three times with PBS-T for 15 minutes each on a shaker. The secondary antibody goat α mouse HRP was diluted 1:20,000 in PBS-T and incubated for 30 minutes at room temperature with gentle mixing. Membranes were again washed three times for 15 minutes each in PBS-T solution. Protein bands were visualized by Millipore Immobilon Western Chemiluminescent HRP Substrate. Images were developed on HyBlot Cl Autoradiography film.

Results

Δ-coil ARNO localization in comparison with full length ARNO

ARFs are lipid-modified at their N-terminus with a myristol group. Therefore ARF activation can only occur at membrane surfaces where this group can insert into the lipid bilayer. ARF-GEFs, such as ARNO, are peripheral membrane proteins, but only the membrane-associated pool is active. To begin understanding the regulation of ARNO during the initiation of epithelial migration, it was important to first understand the sub-cellular localization of MDCK cells infected with a recombinant adenovirus expressing exogenous myc-tagged human full-length ARNO or a recombinant adenovirus expressing exogenous myc-tagged Δ-coils ARNO, in which the coiled coil domain has been removed from the protein. After cell lysis, the cells of each infection were separated into a membrane and cytosolic fraction by centrifugation. The membrane was probed for the ARNO protein and actin and visualized by Western blotting. Figure 2 demonstrates a visible difference between the localization of the wild-type ARNO and the Δ-coils ARNO. In the infection of the cells with virus expressing full length ARNO, there is almost no detectable ARNO present on the membrane. There is, however, a significant amount of ARNO present in the cytosolic fraction. In comparison, in the infection of the cells with virus expressing the Δ-coils ARNO, there are significant and detectable amounts of ARNO in the cytosolic and membrane fractions. This finding is surprising because both full-length and Δcoils ARNO contain the membrane binding PH and polybasic domains. These data suggest that the coiled-coil domain is blocking the membrane binding domains and preventing full-length ARNO from going to the

membrane. Therefore in addition to being a protein-protein interaction domain, the coiled-coil domain may be an auto-inhibitory domain.

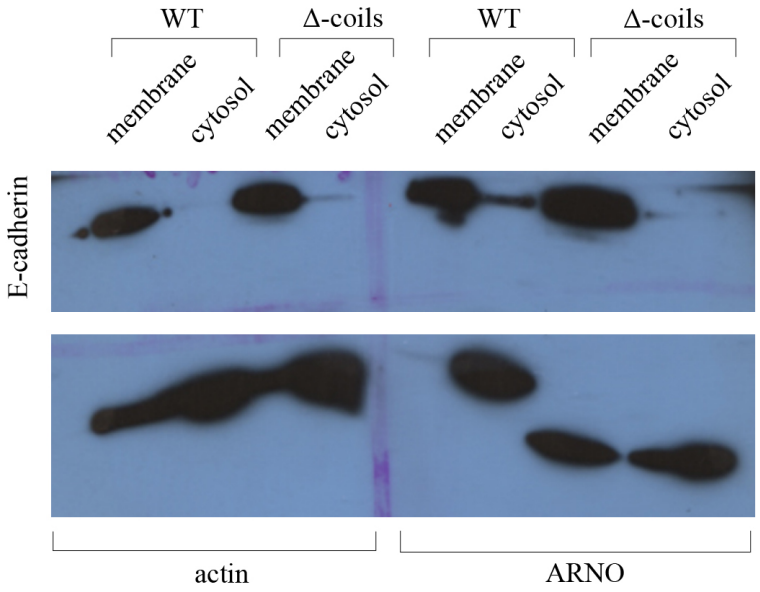

Figure 2: Membrane fractionation demonstrating localization of wild-type ARNO and Δ-coils ARNO. Western Blot analysis was performed on MCDK-T23 cells infected with recombinant adenovirus expressing myc tagged wild-type ARNO and expressing myc tagged Δ-coils ARNO. ARNO was detected with mouse α myc 9E10 primary antibody, actin was detected with mouse α actin and E-cadherin was detected with mouse α E-cadherin.

It is important to note the results of detecting for actin and cadherin as well. E-cadherin is a transmembrane protein, while actin is predominately in the cytosolic fraction. Though the expression varies between the membrane and cytosolic fractions within each construct transfection, the blot displays relatively consistent expression of actin and cadherin between the full length ARNO and the Δ-coils ARNO.

Interaction of coiled coil ARNO and Δ-coils ARNO

Since the coiled-coil domain is blocking association of ARNO with the membrane, we tested the hypothesis that the coiled-coil domain binds to the rest of the protein to physically block membrane binding. GST-tagged Δ-coils ARNO and GST-tagged coiled coil domain of ARNO were grown up in *E. coli*. After isolation and purification of the two proteins, the GST tag of the Δ-coils ARNO was cleaved using thrombin (see Figure 3). The Δ-coils ARNO and GST-tagged coiled coil ARNO were incubated together and the product was observed by SDS-PAGE. A control of incubated Δ-coils ARNO and GST beads was used. The results support the conjectured interaction of the coiled-coil domain with the Δ-coils ARNO in preventing the ARNO from localizing to the membrane but require further confirmation. This is observed in Figure 3. In the lane containing the incubation of the GST-coiled-coil domain with the Δ-coils ARNO, a large band is seen indicating the GST-coiled coil remains. Below this band, a smaller band is seen, displaying a probable binding of the GST-coiled coil ARNO and the Δ-coils ARNO. However, further studies must be completed to confirm the identity of this smaller band. The lanes containing the Δ-coils ARNO and the GST appear to be of about the same length and the lane containing the control also has a band at the same length as the length for the probable binding of the GST-coiled coil ARNO and the Δ-coils ARNO. For this reason, although there is a band supporting the binding of GST-coiled coil to Δ-coils ARNO, there is not enough evidence to show that this is not the identity of the band in the control as well. Though the band displayed by the control is likely GST (25 kDa), the size and quantity of the GST makes it too difficult to distinguish from the results of the incubation of the GST-coiled coil and the Δ-coils ARNO.

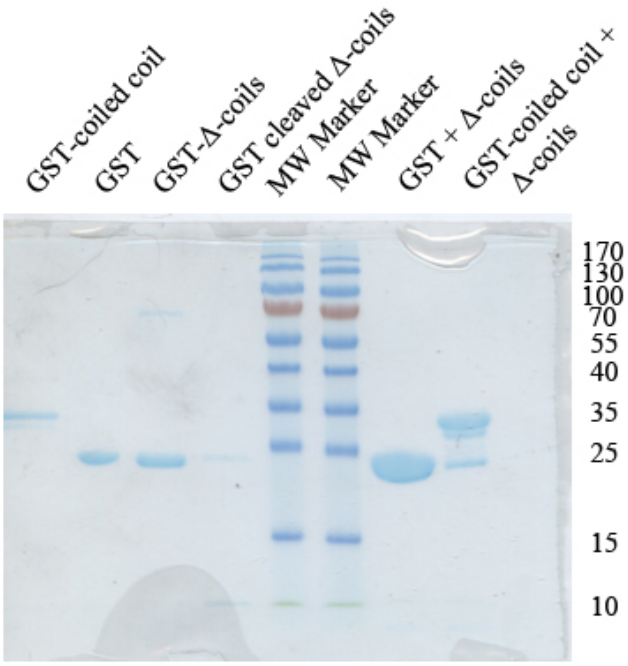

Figure 3: SDS-PAGE gel demonstrating probable interaction between coiled coil ARNO and Δ-coils ARNO. GST-coiled coil ARNO and GST cleaved Δ-coils ARNO were incubated at 4˚ C on a shaker. The starting reactants and products were run on an SDS-PAGE gel along with the starting reactants and products of a control experiment of an incubation of Δ-coils ARNO and GST beads.

Inconclusive results on the interaction of coiled coil ARNO and Δ-coils ARNO

Figure 3 suggests that Δ-coils ARNO binds to the coiled-coil domain. To confirm that it binds to GST-coiled coil and not GST alone, a gel was transferred to a membrane and Western blotted with mouse α ARNO antibody to detect the presence of the GST tagged coiled-coil domain and the Δ-coils ARNO. This was visualized with Western blotting as displayed in Figure 4. The figure displays an unexpected result. While there was a band of the expected size for the coiled coil domain in the lane containing GST-

coiled coil, there also appears to be a strong band just below it that, based on size, seems to be GST. From this observation it is evident that there was a cross-reactivity between the mouse α ARNO antibody and the GST. The GST, used as a control for the experiment in place of GST-coiled coil, are bound by the mouse α ARNO antibody, resulting in a supplementary band. For this reason, a new method for confirming the content of the sample in the gel was pursued.

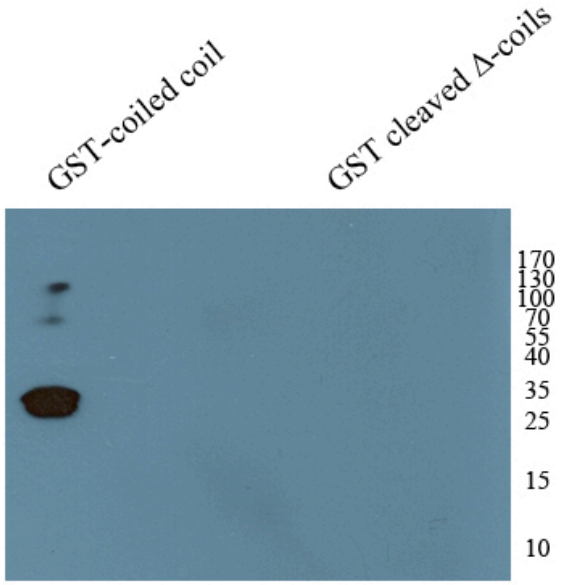

Figure 4: Western blot demonstrating inconclusive results of the presence of an interaction between the coiled coil domain and the Δ-coils ARNO. GST-coiled coil ARNO and GST cleaved Δ-coils ARNO were incubated at 4˚ C on a shaker. The starting reactants and products were run on an SDS-PAGE gel along with the starting reactants and products of a control experiment of an incubation of Δ-coils ARNO and GST beads. The gel was transferred to a nitrocellulose membrane and was detected for the presence of ARNO using mouse α myc primary antibody.

Interaction of coiled coil ARNO and Δ-coils ARNO within new approach

A new approach was used to achieve a successful method of selectively detecting the Δ-coils ARNO and preventing the cross-reactivity of the GST beads and the mouse α

ARNO antibody. In this approach, a Western blot evidencing the interaction of the coiled coil ARNO and Δ-coils ARNO was carried out. MDCK cells were transfected with a recombinant adenovirus system expressing myc tagged Δ-coils ARNO (Altschuler et al., 1999). After lysis, the Δ-coils ARNO was incubated with GST-tagged coiled coil ARNO that had been grown up in *E. coli* and analyzed through Western blotting with the mouse α myc primary antibody. The mouse α myc primary antibody would only recognize the construct that expressed a myc tag, which was only present in a tag on Δ-coils ARNO. Figure 5 demonstrates a clear and distinct band for Δ-coils ARNO in the lane containing the cell lysate and in the lane containing the incubation of the GST-tagged coiled coil ARNO and Δ-coils ARNO. In comparison, there is no detectable protein in the lane which contained the control of GST beads incubated with the Δ-coils lysate.

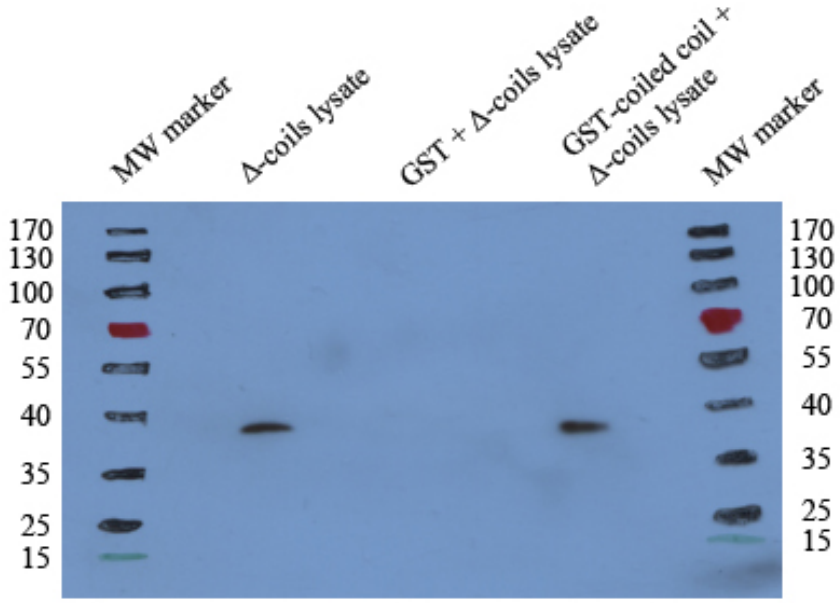

Figure 5: Western blot evidencing the interaction of coiled coil ARNO and Δ-coils ARNO. Δ-coils ARNO isolated from MDCK cells infected with a recombinant adenovirus expressing myc tagged Δ-coils ARNO was incubated with GST-coiled coil ARNO. The product was analyzed through Western blotting, along with a control of an incubation of GST beads and the myc tagged Δ-coils ARNO, using a mouse α myc primary antibody.

IPCEF1 exposure results in down regulation of the coiled coil domain and Δ-coils ARNO interaction

The data described above suggest that the coiled-coil domain of ARNO can act as an auto-inhibitory domain by binding to the rest of the protein. Therefore, in order for ARNO to activate ARFs, other signals must disrupt the auto-inhibitory interaction and allow ARNO to bind to the membrane surface. IPCEF1 has been shown to interact with the coiled coil domain of ARNO (Venkateswarlu, 2003). For this reason, it was hypothesized that IPCEF1 could bind to the coiled coil domain and disrupt the proposed auto-inhibitory interaction. To test this hypothesis, MDCK cells were co-infected with HA tagged IPCEF1 and myc tagged Δ-coils ARNO using a recombinant adenovirus system (Altshuler et al., 1999). This was compared to a control of MDCK cells infected with solely with myc tagged Δ-coils ARNO (Altschuler et al., 1999). After lysis, the Δ-coils ARNO and the product of the co-infection was incubated with GST-tagged coiled coil ARNO that had been grown up in *E. coli*, as well as GST beads to serve as a control. The resulting proteins were analyzed by SDS-PAGE. The resulting gel was visualized through Western blotting separately with the mouse α myc primary antibody and mouse α HA primary antibody. The mouse α myc primary antibody would only recognize the construct that expressed a myc tag, which was only present in a tag on Δ-coils ARNO, whereas the mouse α HA primary antibody would only recognize the construct that expressed an HA tag, which was only present in a tag on IPCEF1. As can be seen in the Figure 6, the infection of the MDCK cells with IPCEF1 resulted in a reduction of the coiled coil domain's interaction with Δ-coils ARNO. In the lane containing the incubation of the Δ-coils ARNO and the GST-coiled coil, there is detectable Δ-coils

ARNO. However, in the lane in which the only addition to the incubation was the IPCEF1, there is no detectable Δ-coils ARNO after incubation with the GST tagged coiled coil and IPCEF1. Furthermore, probing the membrane with mouse α HA primary antibody displayed that IPCEF1 was only present in the cells that had been co-infected with the IPCEF and Δ-coils ARNO.

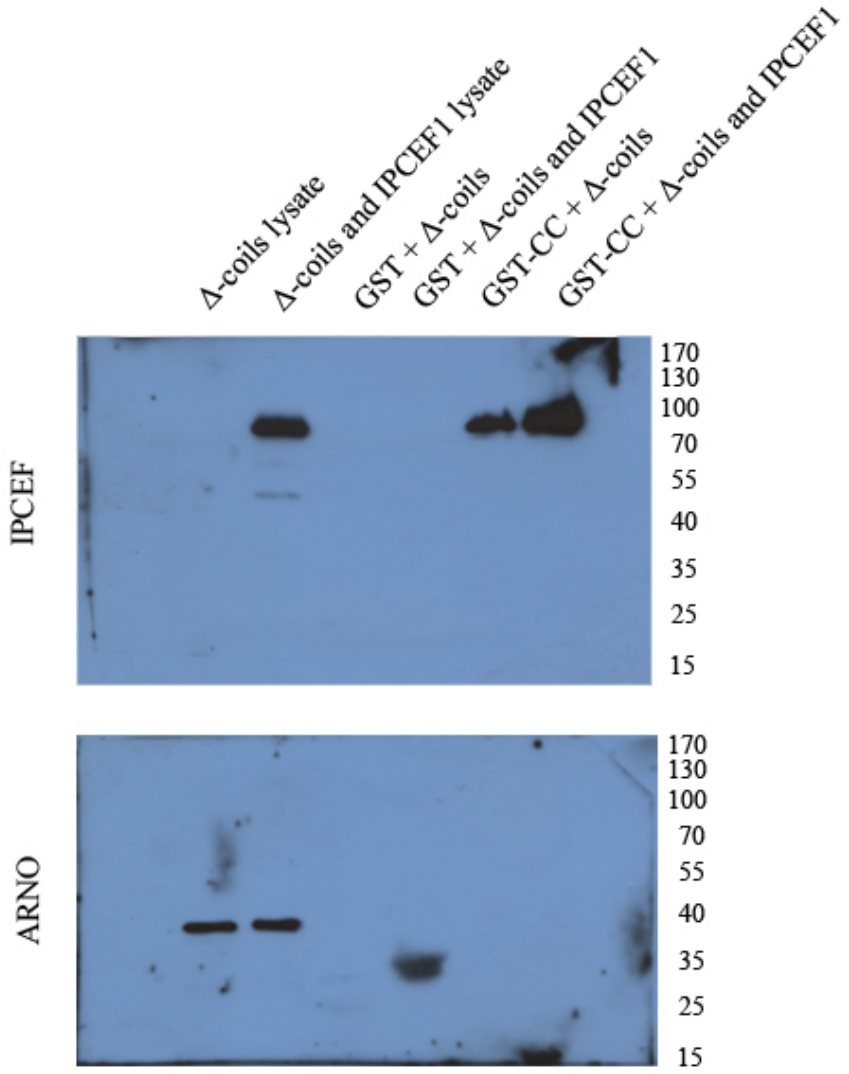

Figure 5: Western blot evidencing the ability of IPCEF to reduce the interaction of coiled coil domain and Δ-coils ARNO. Proteins isolated from MDCK cells infected with a recombinant adenovirus expressing myc tagged Δ-coils ARNO or the myc tagged Δ-coils ARNO and HA-tagged IPCEF were incubated with GST-coiled coil ARNO. The product was analyzed through Western blotting, along with a control of an incubation of GST beads, using a mouse α myc primary antibody, a mouse α HA primary antibody.

Discussion

Experiment Summary

Unexpectedly we found that full-length ARNO is much less prevalent on membranes than is Δcoils ARNO even though both proteins contain the same lipid binding domains. This observation suggests that there is a whole additional layer of regulation of ARNO's activity that has not been appreciated. ARNO may be maintained in the cytosol in an inactive conformation until signals relieve this inhibition. The protein-protein interactions of coiled coil domain are essential in the behavior of ARNO. While many of the factors that interact with the coiled coil domain have been identified, the mechanism by which the interactions result in the activities of ARNO are not well understood. Because of the widespread cell control capabilities, understanding these mechanisms could lead to forms of regulation for important epithelial cell actions. Specifically, this paper deals with the signal transduction pathway resulting in epithelial migration. The downstream effectors of this pathway are ARF6 and Rac1, both of which are regulated by the early actions of ARNO. In addition, both of these proteins are acted upon by ARNO on the membrane, though ARNO is found in the cytosol of unstimulated cells (Shmuel et al., 2006). For this reason, understanding the process by which ARNO localizes from the cytosol to the membrane would outline the step that begins the signal cascade to initiate epithelial cell migration. In particular, it was conjectured that there is an intramolecular interaction within the ARNO protein that contributes and regulates the localization of ARNO.

In this study, it was hypothesized that the coiled coil domain interacts with the

rest of the protein. A recombinant adenovirus system was used to infect MDCK cells with an ARNO construct lacking the coiled coil domain. The coiled coil domain was produced through expression in *E. coli* cells. Once expressed, these proteins were incubated together to assay the ability of the two proteins to interact. The products of the incubations were analyzed through SDS-PAGE and further by Western blotting using antibodies targeting the corresponding tags included in the constructs. These results were visualized with Western blotting, displaying data that supported the presence of an interaction within the ARNO protein between the coiled coil domain and the remaining protein. In addition, co-expression of IPCEF1, a protein known to interact with the coiled coil domain, disrupted this interaction.

Δ-coils ARNO interacts with the coiled coil domain to retain ARNO in the cytosol

The PH and PB domains of ARNO cooperate to bring ARNO to membrane surfaces. The results showing that full-length ARNO is less prevalent on membranes and showing that the coiled coil domain interacts with the rest of the protein suggest that the coiled coil domain can block these domains. It was predicted that an intraprotein interaction was responsible for the retention of ARNO in the cytosol in unstimulated cells. The data from this study supports this prediction, prompting the production of a model for the configuration of ARNO in the cytosol. In the model, it is suggested that the coiled coil domain folds over the rest of the ARNO protein, thereby preventing the PH domain from interacting with membranes. Cell migration has been shown to be initiated by several growth factors (Hay, 2005). For this reason, it is proposed in the model that once the appropriate growth factors are added to induce epithelial cell migration, the

ARNO protein unfolds and ARNO moves to membrane surfaces. A summary of this model can be seen in Figure 7.

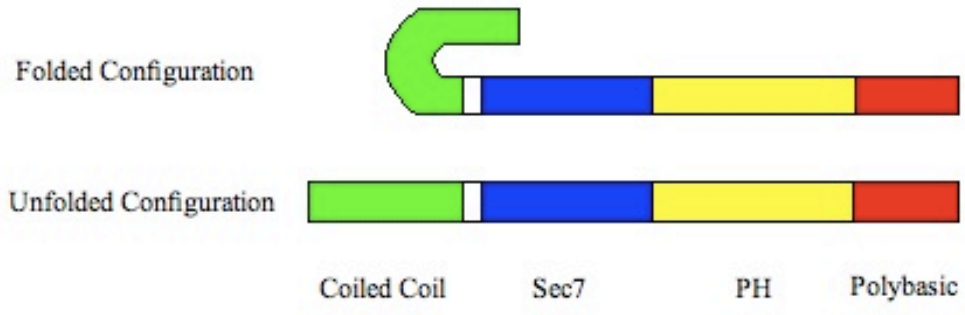

Figure 7: Model of ARNO configuration in cytosol. For ARNO to remain in the cytosol, the coiled coil domain must be inhibited as displayed in the folded configuration. For ARNO to localize to the membrane, the coiled coil domain must be available for interaction with scaffold proteins as displayed in the unfolded configuration.

Role of coiled coil domain in the localization of ARNO to the membrane

Use of the Δ-coils ARNO, or the absence of the coiled coil domain in ARNO, in the infection of MDCK cells resulted in unspecific localization. Previous studies have supported this conjecture (Shmuel et al., 2006), bolstering this reasoning to use as a foundation for later experimentation. In addition to acting to blocking membrane binding in the absence of appropriate signals, the coiled coil domain may promote the localization of ARNO to particular sub-cellular regions by interacting with other proteins. One of the protein-protein interactions of the coiled coil domain is known to be the association with IPCEF1. IPCEF1 is known to be a scaffold protein that allows for the recruitment of ARNO to the plasma membrane (Venkateswarula, 2003). Furthermore, IPCEF1 is

required for HGF induced Arf6 activation suggests that it plays the role of recruiting cytohesin in response to upstream signals (Attar et al., 2010).

Data from the Santy lab suggests that scaffolds that bind to the coiled coil domain of ARNO also promote proper signal transduction downstream of ARNO. While there is no previous literature outlining the mechanism by ARNO, a possible explanation lies in a previously proposed model. In the model, it is predicted that ARNO acts on Rac1 through the DOCK180/Elmo complex (White et al., 2010). Furthermore, the model suggests interaction of ARNO and the DOCK180/Elmo complex is in a coiled coil domain dependent fashion. The coiled coil domain would be acting as a scaffolding protein and be responsible for assembling the DOCK180/Elmo complex. (White et al., 2010). Previous studies have shown that this association is aided by IPCEF1 and GRASP (White et al., 2010). Because this model deals directly with Rac1, it is further proposed that the model applies to solely this signal transduction pathway and does not describe the mechanism of the ARF6 signal transduction pathway. Though both are required effects in order for epithelial cell migration to be initiated, they need not be regulated together (White et al, 2010).

IPCEF1 competes for with Δ-coils ARNO the coiled coil domain

The model summarized in Figure 7 suggest that upstream signals will disrupt the interaction of the coiled coil domain with the rest of ARNO. This is supported by the results achieved in this study. The presence of IPCEF1 with the coiled coil domain and the Δ-coils ARNO resulted in an inability of the coiled coil domain and Δ-coils ARNO to bind. Though the region of the Δ-coils ARNO that interacts with coiled coil domain is

currently unspecified, the disruption caused by IPCEF1 suggest that the region in which the Δ-coils ARNO binds the coiled coil domain is the same or is an overlapping region in which IPCEF1 binds. This could explain the ability of IPCEF1 to inhibit the intraprotein interaction. It also suggests that the interaction of the coiled coil domain and IPCEF1 is stronger than that of the Δ-coils ARNO and the coiled coil domain. In addition, this explanation for the inhibiting activity supports the proposed model of the folding of ARNO to remain in the cytosol. Configuration changes of a protein are often unstable and would therefore cause a protein to favor alternative binding.

Further investigations

There is still much to be learned about the signal transduction pathways involving ARNO that result in a migratory phenotype. To branch upon this study, truncated versions of the Δ-coils ARNO are currently being assessed using a similar approach as in this study. The intention is to isolate the region within Δ-coils ARNO that interacts with the coiled coil domain. Results from this could provide more support for the model proposing the folding of the ARNO protein and would allow for initial studies in creating methods of targeting the interaction site for regulation. For example, inhibition of the interaction between the region on ARNO and the coiled coil domain could make the coiled coil domain more readily accessible, thereby increasing cell migration in wound healing. The importance of this cell activity supports the role of ARNO and the interest in conducting more precise studies of its regulation.

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Notable Courses

Biochemistry and Molecular Biology I, II and III with Labs
Organic Chemistry I and II with Lab
Chemical Principles I and II with Labs
Microbiology with Lab
Mechanical Physics with Lab
Electrical and Magnetic Physics with Lab
Principles of Immunology

Honors, Awards & Achievements

Phi Eta Sigma Honor Society - *Inducted Spring 2009*
National Society of Collegiate Scholars- *Inducted Spring 2009*
Dean's List- *Fall 2008, Fall 2009*
Scholarship for Study Abroad in Italy- *Summer 2009*
Edward B. Nelson Summer Research Grant- *Summer 2011*

Relevant Experience

Dr. S. Blair Hedges' Lab *October 2008-May 2009*
Research Assistant
Conducted an independent project investigating the phylogenetic relationship of a genus of South American Lizards using several techniques, including:

- DNA extractions
- PCR and Gel Purification
- Sequence analysis using MEGA

Albert Einstein Medical Center *August 2009*
Assisted in collecting data for a clinical research project in patients infected with hepatitis C virus

Alliance Pharma *June 2010-August 2010*
Summer Intern
Conducted experimentation to determine a technique for the quantification of Exenatide in dry blood spot using competitive ELISA. Also aided in other contract work, including:

- Cell Based Analysis with Meso Scale Discovery Equipment
- BSA Assays using Thermo Scientific Pierce BCA Protein Assay Kit

Dr. Lorraine C. Santy's Lab *January 2010-present*
Research Assistant
Initiated an independent project to determine the structure of the chimeric region of a protein involved in the activation of GTPase regulation of epithelial cells. The techniques employed include:

- Cell culture (MDCK epithelial cell line)
- Western Blots

Dr. Laurie B. Kile's Psychiatry Practice *April 2011- December 2011*
Assistant
Aided Dr. Laurie B. Kile in preparing records for patients, overseeing the office duties and communicating with healthcare companies for patients

Extracurricular Activities

Schreyer Honors College Mentor *Fall 2009 and Fall 2010*
Provide guidance to incoming Schreyer Honors College students regarding academics, housing and student life

University Park Undergraduate Association Programming Committee
Secretary *September 2009-May 2010*
Recorded minutes of each meeting with the Programming Committee of the University Park Undergraduate Association

THON *September 2010-present*
Rules and Regulations Captain
Organized security and logistics for a national, year-long fundraiser benefiting pediatric cancer culminating in a 46 hour dance marathon

Lion Ambassadors *December 2010-present*
Be a Part From the Start Chair
Aid the Penn State Alumni Association in upholding the history and tradition of the University through planned events and prospective student tours