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THE EFFECT OF RHO FAMILY PROTEINS ON HGF TO ARF6 SIGNALING

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Abstract

Epithelial cell migration is a coordinated process that requires precise polarization in order to result in productive movement. GTPases are among the proteins that regulate motility. This thesis looks at the interaction between signaling processes of two families of GTPases involved in cellular motility, Rho and Arf. We use co-immunoprecipitation experiments to show that RhoA-GTP, but not other members of the Rho family, interacts with an intermediate in the signaling events leading to Arf6 activation. We then investigate the effects of RhoA interaction with the amount of Arf6 activation using GGA3 pulldown assays. Lastly, one potential molecular mechanism through which RhoA could exert an influence on the signaling pathway is tested with co-immunoprecipitation experiments.

Acknowledgements

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Introduction

Cell movement is an important characteristic of life, essential to the life cycle of organisms ranging from bacteria to humans. In higher eukaryotes, epithelial cell migration underlies normal growth and development processes such as tissue morphogenesis and wound healing, along with contributing to the progression of cancer through tumor cell metastasis. A cell must polarize itself in order to move productively in one direction. Intracellular signaling establishes this polarity so that migration can occur. When movement begins, the cell first extends protrusions due to the reorganization of actin polymers. If the actin is rearranged into a "dendritic" network, broad lamellipodia are formed. Conversely, spike like filopodia can be formed from a parallel arrangement of actin bundles. The protrusions then adhere to the extracellular matrix through integrin activation, and are used for traction to pull the cell body forward. Once the cell moves over the adhesions, they are disassembled at the rear [1].

Motility is tightly regulated by the cell and under normal conditions responds to extracellular stimulation from growth factors. A potent growth factor that induces many epithelial cells to migrate is hepatocyte growth factor (HGF), also known as scatter factor. HGF signals through the c-Met tyrosine kinase receptor, which activates the GTPase Ras as an early consequence of HGF binding [2]. Ras has a well characterized role in cellular growth and transformation; however, its function in migration is less certain. Other members of the Ras superfamily of small GTPases are known to regulate cell motility and may function as effectors or regulators of the Ras signaling cascade. The work in this thesis helps to define an unknown signaling pathway that regulates cellular polarity during epithelial cell migration.

GTPases and their role in motility

Guanosine triphosphatases (GTPases) act as binary molecular switches, active when bound to GTP and inactive upon a conformational change after hydrolysis to GDP. GTPases have low intrinsic hydrolysis and exchange capabilities, which enable them to be regulated by two classes of proteins. Guanine nucleotide exchange factors (GEFs) initiate exchange of bound GDP for GTP, thus activating GTPases, whereas GTPase-activating proteins (GAPs) inactivate the proteins by promoting hydrolysis the bound GTP [3].

The Ras superfamily of small GTPases is highly involved in intracellular signaling pathways, including those involved in migration. It is historically known for the founding member, Ras, and its infamous role in cancer. The family has over 150 members which all share a conserved GTP binding domain [3].

One subgroup within the Ras superfamily is the Rho family (Ras homology) of GTPases. The members of this family are known to regulate cell polarity, migration, adhesion, and cell cycle progression [3]. The well characterized members include Cdc42, Rac1, and RhoA. Activated at the front of cells, Cdc42 regulates cell polarity by locating the MTOC (microtubule organizing center) and Golgi in front of the nucleus [4], as well as restricting where lamellipodia can form [1]. Rac1 is also active at the front of migrating cells, where it is thought to induce actin polymerization leading to the formation of protrusions [4]. A recent study has shown that Rac1 forms actin rich brushes in zebra fish embryos, which play an essential role in germ cell migration [5].

Three proteins, RhoA, RhoB, and RhoC, comprise the more specific Rho subfamily of GTPases. Among their effector proteins, two of the best studied are the serine/threonine kinase, ROCK (Rho-associated coiled-coil forming kinase/Rho kinase), and mDia (mammalian homolog

of Drosophila diaphanous). These two proteins cooperate to regulate contraction in the cell. mDia is a formin protein that leads to long and straight actin polymerization. ROCK can promote actomyosin contraction in two ways. It can activate myosin light chain through phosphorylation, and also phosphorylate myosin phosphatase, thus inactivating a myosin inhibitor [4].

The research on mDia and ROCK formulated the idea that Rho is involved in contracting the cell rear. RhoA was specifically implicated in this process through the observation that inhibition of RhoA leads to an extended tail [1]. Interestingly, more recent work has also uncovered RhoA activation as a sharp band at the leading edge of protrusions during migration [6], [5]. Compared to RhoA, much less is known about the normal functions of RhoB and RhoC in the cell. RhoB is known to localize to endosomes, where it promotes the formation of an actin coat through recruitment of mDia [4], [7]. RhoC was identified as an overexpressed gene in a highly metastatic melanoma selection assay [8]. Its *in vivo* functions remain elusive, as knockout mice are viable and RhoC is nonessential for the maturation and migration of hematopoietic cells [9]. However, RhoC knockout Mouse Embryonic Fibroblast (MEF) cells show abnormal stress fiber formation, implicating RhoC in the regulation of actin [9].

Dysfunction and abnormal regulation of small GTPases often contributes to the pathogenesis of cancer. The transforming potential of oncogenic Ras is well known, but proteins in the Rho family have a dissimilar effect on the development and progression of cancer than that of Ras. Overexpression of RhoA, Rac1, or Cdc42 is insufficient to produce transformation comparable to overexpressing Ras; however, all three are required for Ras induced transformation [4].

Rho GTPases may instead exert their effect on cancer by promoting metastasis. As the cause of about 90% of cancer related deaths, metastasis is a crucial concern for treatment of the disease [10]. Since motility is essential for cancer metastasis [10], it seems logical that Rho proteins could influence this process. Indeed, overexpression of RhoA and RhoC is often found in clinical cancers [4]. For example, higher levels of RhoA correlate with more advanced stages of breast carcinoma, which are more invasive and thus metastatic, and RhoC is found to be overexpressed in both inflammatory breast cancer and invasive ductal carcinomas [10]. RhoC can be a valuable biomarker for breast cancer prognosis since its expression increases with progression of the disease [11]. The fact that RhoC knockout mice show decreased metastatic potential [9] indicates that it could be a potential target for cancer therapies.

Another family of GTPases that regulate cellular processes is the ADP ribosylation factors (Arfs). Their main role is to regulate membrane trafficking along the endosomal and secretory pathways. All Arf proteins are ubiquitously expressed and highly conserved among eukaryotes, and contain a myristoylation near the N-terminus, which apparently functions to anchor them to membranes [12]. Class I Arfs (Arf1, Arf2, and Arf3) regulate the assembly of coat complexes in budding vesicles along the secretory pathways. Arf1, the best studied of Class I members, induces clathrin recruitment to the late Golgi in part by recruiting GGA (Golgi-localized gamma-ear-containing Arf-binding) proteins, and regulates Golgi structure by promoting spectrin and actin assembly on Golgi membranes [12]. Class II Arfs (Arf4, Arf5) also play a role in Golgi transport, although less is known about the details of their functions [12].

As the sole member in Class III, Arf6 is the least conserved and is involved in slightly different processes. One of its major functions is to regulate endocytosis, including both clathrin dependent and independent pathways, and endosome recycling [12]. Arf6 also regulates actin

polymerization, and thus affects the motile potential of cells. Arf6 has been shown to be required for the formation of actin rich protrusions and membrane ruffles [13]. Indeed, expression of a dominant negative Arf6 mutant in Madine Darby Canine Kidney (MDCK) cells, a suitable model epithelial cell line, inhibits migration in a wound healing assay and in response to growth factor [14], [15].

Identifying the pathway to Arf6 activation

There is evidence to suggest that Arf6 may be activated as a consequence of Ras signaling. Since HGF is a potent growth factor that stimulates epithelial cells to migrate, it is used as an *in vitro* model to study these processes [16]. HGF signals through the c-met receptor which activates Ras, and it is also known to activate Arf6 [15]. Certainly this correlation is not conclusive, yet it merits investigation because the pathway is currently undefined and may be novel to Ras signaling.

Like all GTPases, Arfs require GEFs to catalyze the exchange of GDP for GTP to induce a conformational change to the active form. There are several classes of Arf GEFs, all of which contain a conserved catalytic Sec7 domain. Some of the Sec7 GEFs are localized to the Golgi, while the cytohesin class can be recruited to the plasma membrane following phosphatidylinositol 3 (PI3)-kinase signaling [17]. Among the cytohesins, cytohesin 2/ARNO has specifically been linked to the role of Arf6 in cell migration. Via its PH domain, cytohesin 2 binds Arf6 in a GTP dependent manner [18]. Overexpression of cytohesin 2 elevates Arf6-GTP levels and increases the migratory activity of MDCK cells [14]. These changes are similar to those created after HGF exposure.

Cytohesin 2 also binds another protein known as Ipcef/Pip-3E. Ipcef was identified separately in both a yeast two hybrid screen as a cytohesin binding protein [19] and a screen for phosphoinositide binding proteins [20]. Ipcef binds the coiled-coil domain of cytohesin 2 through its C terminus and is localized to the plasma membrane following growth factor stimulation in a cytohesin 2 dependent manner. More specifically, Ipcef enhances the GEF ability of cytohesin 2 to induce Arf6 activation both *in vitro* and *in vivo* [19].

Ipcef is likely the C-terminal half of CNK3, a scaffold protein belonging to the connector enhancer of ksr (CNK) family. Ipcef is homologous to the C terminal ends of human CNK1 and CNK2, and CNK3 is identified as encoding a protein homologous to the N-terminal half of CNK1 and CNK2 [21]. If CNK3 and Ipcef were linked together, they would create a protein similar to the other CNKs.

Dr. Lorraine Santy has preliminary unpublished data to support the claim that human CNK3 and Ipcef are mis-annotated and are in fact a single gene producing a protein similar to the other CNK family members. RT-PCR has identified a spliced RNA transcript in human cells containing sequences from both Ipcef and CNK3. Additionally, siRNA knockdown of either CNK3 or Ipcef sequences decreases levels of the CNK3 protein detected with a commercial antibody. This protein is also implicated in cell motility by the fact that siRNA to Ipcef reduces MDCK migration after HGF exposure.

The experimental evidence that the Arf6 GEF, cytohesin 2/ARNO, interacts with a protein in the CNK family suggests this pathway links to Ras signaling. CNK was originally identified in Drosophila as a connector protein that enhanced the action of ksr, a kinase downstream of the Ras pathway, and had the ability to bind to Raf [22]. Further studies showed

that *Drosophila* CNK acts as a scaffold protein to regulate Raf to Mek signaling in the MAPK cascade downstream of Ras [21].

Acting as scaffold proteins, CNKs have several conserved protein interaction domains. Listed from the N-terminus, the SAM (sterile alpha motif) domain can homo/hetero-oligomerize, bind RNA sequences, or interact with membrane lipids. The CRIC domain stands for conserved region in CNK and consists of 80 amino acids that are potentially involved in the binding of other proteins. PDZ is a domain that can bind target proteins or interact with other PDZ domains or phosphoinositides. A PH domain begins the sequence for Ipcef, which binds phosphoinositides to serve as membrane anchorage. Lastly, CNK contains a CRAC domain, an acronym for conserved region among chordates since it is not found in *Drosophila* or *C. elegans* [23].

CNK proteins have been implicated in multiple GTPase signaling pathways, including Ras, Rho, Rac, Ral, and Arf [23]. One function they appear to serve is linking the signaling activities of Ras and Rho. Through its PH domain, CNK1 binds RhoA in a GTP dependent manner [24]. HGF signals through Ras, and active RhoA has been shown to inhibit HGF induced migration [25]. The method by which Rho regulates cellular motility has yet to be definitively determined, though.

RhoA is thought to be involved in contracting the rear of migrating cells since inhibition of RhoA leads to the formation of an extended tail [1]. This observation correlates well with a study that showed RhoA is active at the back and sides of migrating cells [26]. In light of these data, Dr. Santy hypothesizes that RhoA helps establish cell polarity during migration by inhibiting the activation of Arf6 at the back and sides of the cell, thus limiting it to the leading edge where it has been shown to be active [27].

The aim of this thesis is to investigate the involvement of Rho family GTPases with the signaling cascade leading to Arf6 activation. To determine which Rho family members interact with the pathway, co-immunoprecipitation assays were performed using Ipcef as bait to test Rho binding. Once it was shown that RhoA-GTP binds Ipcef, whereas other notable members of the Rho family, Rac1, Cdc42, RhoB, and RhoC, do not, we looked into possible effects of RhoA binding. Pulldown assays that detect the amount of active Arf6 in the cell were used to analyze the effect of constitutively active RhoA on HGF induced Arf6 activation. We hypothesized that RhoA would decrease the levels of Arf6 activation, and although it is not conclusive, preliminary data may support this idea. Lastly, one possible mechanism through which RhoA might exert influence on the Arf6 pathway was investigated. Co-immunoprecipitation experiments determined that RhoA and cytohesin 2/ARNO can simultaneously bind Ipcef, suggesting RhoA does not prevent the binding of cytohesin 2 to Ipcef.

Results

RhoA has been previously shown to bind the PH domain of CNK1 in a GTP dependent manner [24]. We were interested in whether RhoA could also interact with CNK3 in this way to act as a possible regulator of the pathway to Arf6 activation. To test this possibility, we used co-immunoprecipitation experiments in human epithelial cells as an *in vivo* binding assay. As described earlier, Ipcef is most likely the C-terminal half of CNK3 and it contains a PH domain homologous to the one on CNK1, so if RhoA were to bind CNK3, it would likely be through the PH domain in Ipcef. Therefore, we could use Ipcef to test RhoA binding since the full length CNK3 has yet to be cloned.

Along with RhoA, two other major members of the Rho GTPase family, Rac1 and Cdc42, were assayed for interaction with Ipcef. HeLa cells were transfected with HA-Ipcef and constitutively active mutants of myc tagged RhoA, Cdc42, or Rac1. HA-Ipcef was then immunoprecipitated and the resulting fractions were analyzed for the presence of Rho protein via the myc epitope. As shown in the Western blot in Figure 1, RhoA-GTP co-immunoprecipitated with Ipcef, while Cdc42-GTP and Rac1-GTP did not co-immunoprecipitate. This result suggests that RhoA has a unique ability among Rho family members to interact with Ipcef, and thus likely CNK3.

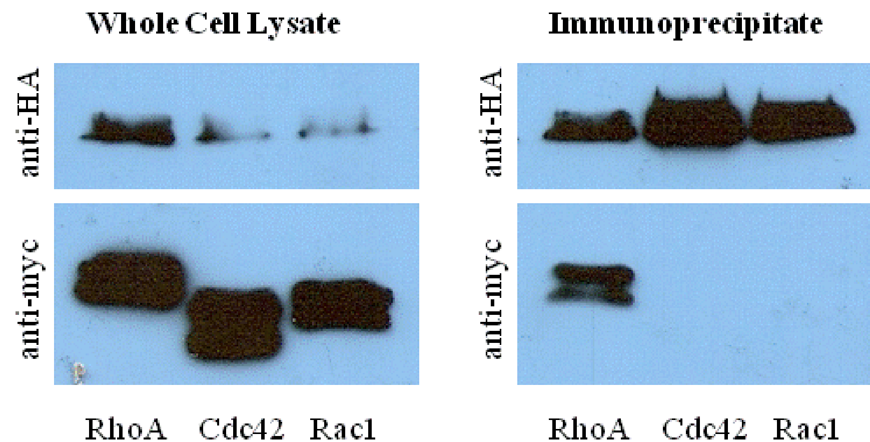

Figure 1. **RhoA, but not Cdc42 or Rac1, binds Ipcef.** HeLa cells were co-transfected with HA-Ipcef and myc-RhoA-Q63L, myc-Cdc42-Q61L, or myc-Rac1-Q61L. After 24 hours, cells were lysed and cleared of cellular debris. HA-Ipcef was immunoprecipitated with rabbit anti-HA. Fractions from the whole cell lysate and immunoprecipitate were run on a 13% SDS-PAGE gel, transferred to nitrocellulose, and Western blotted with mouse anti-HA or mouse anti-myc.

As the names suggest, RhoA is more closely related to RhoB and RhoC than Rac1 and Cdc42. It is possible then, that CNK binding is a characteristic feature of the RhoA subfamily proteins. To test this possibility, co-immunoprecipitation experiments similar to the ones above were performed with RhoB and RhoC. Figure 2 clearly shows that RhoA can immunoprecipitate with Ipcef, whereas RhoB and RhoC can not. This evidence more specifically identifies an exclusive role for RhoA in the signaling pathway resulting in Arf6 activation.

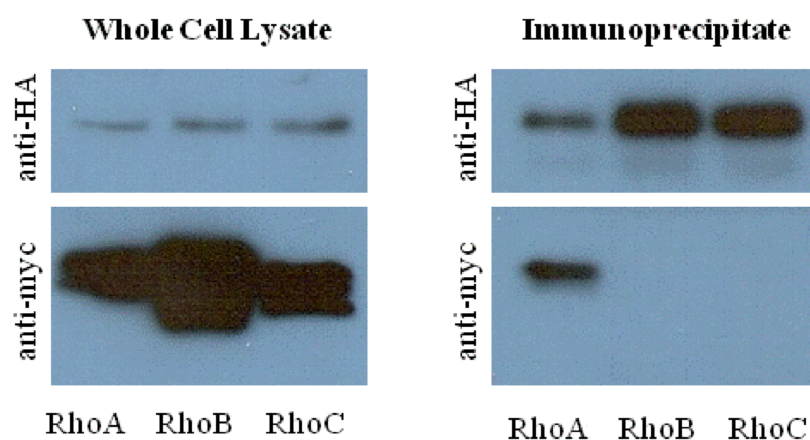

Figure 2. **RhoA shows unique binding to Ipcef among Rho subfamily proteins.** HeLa cells were co-transfected with HA-Ipcef and myc-RhoA-Q63L, myc-RhoB-G14V, or myc-RhoC-G14V. After 24 hours, cells were lysed and cleared of cellular debris. HA-Ipcef was immunoprecipitated with rabbit anti-HA. Fractions from the whole cell lysate and immunoprecipitate were run on a 13% SDS-PAGE gel, transferred to nitrocellulose, and Western blotted with mouse anti-HA or mouse anti-myc.

Constitutively active mutants of the Rho proteins were used to ensure that the overexpressed proteins were mainly in the GTP bound state. However, the altered amino acids in the mutants are not identical. The constitutively active RhoA used in Figure 1 and 2 contains a glutamine to leucine mutation at the 63rd amino acid. The Rac1 and Cdc42 constructs also contain glutamine to leucine mutations at an analogous position, but the RhoB and RhoC constructs have glycine to valine mutations at a separate position in the 14th amino acid residue. In order to confirm the above results, the G14V mutation, identical to the one in RhoB and RhoC, was introduced into a wild type RhoA construct via site directed mutagenesis.

After creating the RhoA-G14V mutation, we could then comparatively asses the Ipcef binding capabilities of the Rho subfamily proteins more definitively. The same co-immunoprecipitation experiment as in Figure 2 was performed with RhoA-G14V to determine if the different mutation affected the binding to Ipcef. As shown in Figure 3, RhoA-G14V retains the ability to interact with Ipcef, while RhoB-G14V and RhoC-G14V do not exhibit this property. When the amount of RhoA appearing in the immunoprecipitate is compared to the total expression levels, it is apparent that RhoA-Q63L shows significantly better ability to interact with Ipcef than does RhoA-G14V. It is possible that this discrepancy is due to differences in the functional characteristics of the two activating mutations, which has been observed in other studies.

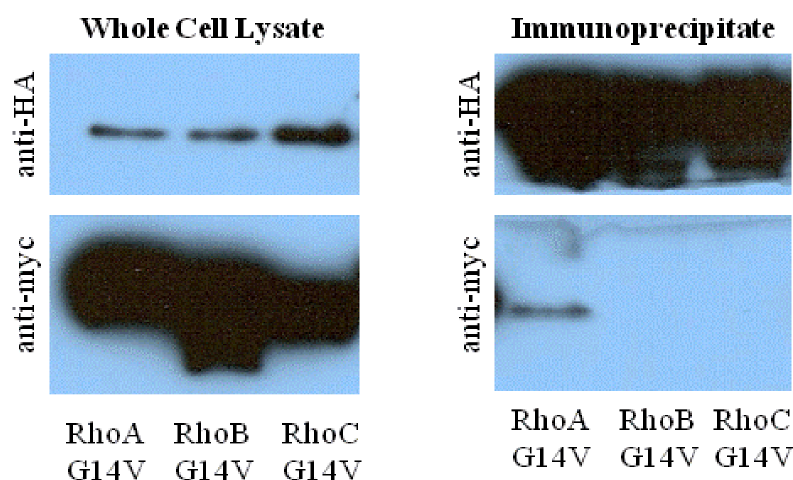

Figure 3. **RhoA-G14V also binds Ipcef while RhoB-G14V and RhoC-G14V do not.** HeLa cells were co-transfected with HA-Ipcef and myc-RhoA-G14V, myc-RhoB-G14V, or myc-RhoC-G14V. After 24 hours, cells were lysed and cleared of cellular debris. HA-Ipcef was immunoprecipitated with rabbit anti-HA. Fractions from the whole cell lysate and immunoprecipitate were run on a 13% SDS-PAGE gel, transferred to PVDF, and Western blotted with mouse anti-HA or mouse anti-myc.

Once the interaction with Ipcef was determined, the next step was to uncover the impact of RhoA on the signaling pathway leading to Arf6 activation. We hypothesized that active RhoA inhibits signaling in this cascade by binding to Ipcef, which would serve as a mechanism to regulate the spatial organization of Arf6 activity. Testing this hypothesis requires a method to analyze the amount of active Arf6 in the cell. We used an assay developed by Dr. Santy that utilizes the ability of GGA3 to bind only active Arf6 and not the GDP bound conformation. By fusing GGA3 to sepharose beads via GST, the GGA3 beads can be used to specifically "pull down" only Arf6-GTP in a manner similar to immunoprecipitation.

The T23 line of MDCK cells were used for this experiment because they are a suitable model for HGF induced epithelial cell migration [16]. The cells were reverse transfected in duplicate with pCB7 empty plasmid as a control or RhoA-Q63L. After being serum starved overnight to reduce growth factor signaling, HGF was added to one of the plates containing mock transfected cells, and one of the plates with RhoA-GTP expressing cells. The cells were lysed while they still showed free edges, within 4-6 hours after HGF exposure, and analyzed for the amount of active Arf6 using the GGA3 pulldown assay.

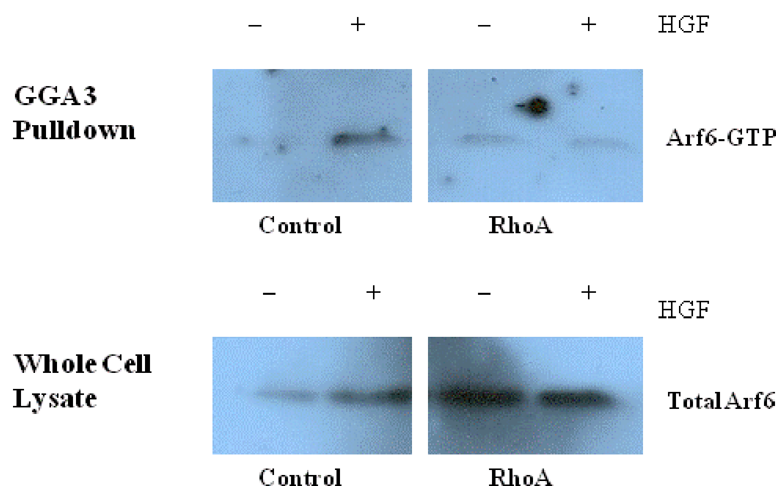

Figure 4. **Effect of RhoA-GTP on HGF induced Arf6 activation.** MDCK-T23 cells were reverse transfected with myc-RhoA-Q63L or pCB7 as a control. The media was changed to low serum, 1% FBS DMEM 3-4 hours later. The next morning, HGF was added to the indicated cells. The cells were lysed before becoming confluent (within 4-6 hours), cleared of cellular debris, and incubated with GGA3-GST-sepharose beads. Fractions from the whole cell lysate and GGA3 pulldown were run on an SDS-PAGE gel, transferred to PVDF, and analyzed by Western blotting with rabbit anti-Arf6.

Figure 4 shows an example of one such experiment. An Arf6 specific antibody was used for Western blotting, which has no preference for active versus inactive Arf6. Therefore, the bands from the whole cell lysate represent total endogenous Arf6 levels. The GGA3 pulldown lanes represent only active Arf6-GTP. To determine to extent of Arf6 activation, the relative levels of active Arf6 to total Arf6 must be compared between the HGF exposed and unexposed cells. Focusing on the pCB7 transfected control lanes, it appears that the cells incubated with HGF show relative Arf6 activation. However, the same is not true for the RhoA expressing cells, which show approximately equal levels, suggesting that RhoA is hindering HGF induced Arf6 activation. This result is still preliminary and more research is ongoing to strengthen the data.

We were also interested in the immediate molecular effect RhoA has on the signaling complex organized by CNK3. One potential consequence of RhoA-GTP binding is that it prevents the interaction of ARNO/cytohesin 2 with CNK3. We hypothesized that this scenario might be a way for RhoA to limit Arf6 activation to the leading edge. Since the Ipcef portion of CNK3 has both the ability to bind ARNO [19]and as we have shown, RhoA-GTP, we tested whether these binding events were mutually exclusive.

To determine if RhoA-GTP could inhibit ARNO from binding to Ipcef, we performed co-immunoprecipitation assays in HeLa cells expressing Ipcef along with both RhoA and ARNO. As shown in the first two lanes in Figure 5, immunoprecipitating Ipcef also precipitates ARNO. If RhoA-GTP interfered with the binding of ARNO and Ipcef, the levels of ARNO in the immunoprecipitate should be decreased during co-expression of active RhoA. However, the last two lanes show that there is no change in the ability of ARNO to bind Ipcef with the expression of either wild type or constitutively active RhoA. This evidence suggests that RhoA is not limiting ARNO to Ipcef binding. It should be noted though, that Ipcef is only the c-terminal portion of CNK3. It is possible that interactions with the full length protein would differ from the results obtained here.

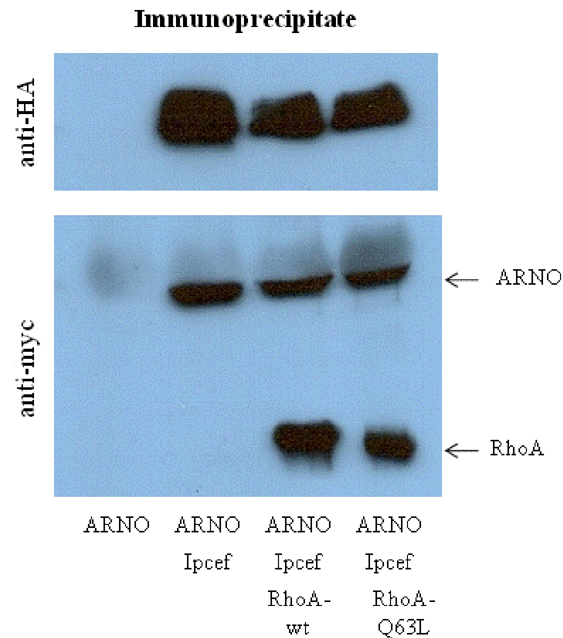

Figure 5. **RhoA-GTP and ARNO can simultaneously bind Ipcef.** HeLa cells were transfected with either a) myc-ARNO, b) myc-ARNO and HA-Ipcef, c) myc-ARNO, HA-Ipcef, and myc-RhoA-wt, or d) myc-ARNO, HA-Ipcef, and myc-RhoA-Q63L. pCB7 was included in a) and b) to maintain a constant amount of DNA throughout the transfections. After 24 hours, cells were lysed and cleared of cellular debris. HA-Ipcef was immunoprecipitated with rabbit anti-HA. The immunoprecipitate was run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and Western blotted with mouse anti-HA or mouse anti-myc.

Discussion

The processes that regulate epithelial cell motility continue to be an ongoing body of substantial research. Investigating cellular migration is important to both understanding normal development and uncovering mechanisms relating to cancer metastasis. This thesis dealt with the regulatory processes that set up and maintain cellular polarity during migration. It focused on the signaling pathway leading to the activation of a low molecular weight GTPase, Arf6, which is known to play a role in motility through its effect on the actin cytoskeleton [14], [15], [13]. We examined the interaction between the Rho family of GTPases and Arf6 activation using a molecular approach to gain insight into the cellular processes governing migration.

Co-immunoprecipitation experiments demonstrated that RhoA can bind to Ipcef, but RhoB, RhoC, Rac1, and Cdc42 lack this ability. Keeping in mind that Ipcef is likely the C-terminal half of CNK3, we have shown that RhoA interacts with another CNK family member in addition to CNK1 [24]. From the data presented here, it cannot be absolutely determined that the other Rho family proteins do not interact with full length CNK3. However, it seems unlikely such would be the case due to the fact that RhoA binds the PH domain of CNK1 [24], which is homologous to the PH domain on Ipcef, and therefore is highly likely to be the site of RhoA binding. It would be expected that the other members share the same binding region, and because they do not bind Ipcef, it would imply that they would also fail to bind full length CNK3.

Interestingly, there appears to be a difference in the amount of RhoA interacting with Ipcef between the two activating mutations, although confirmation awaits a comparative analysis within a single experiment. Both mutations are in the nucleotide binding pocket of RhoA where they interfere with the hydrolysis of GTP and the overall structure of the two mutants is similar.

However, it has been demonstrated that the Q63L mutant retains a higher ratio of GTP/GDP bound isoforms than the G14V mutant, which probably also accounts for the observation that RhoGDI binds RhoA-Q63L much less than RhoA-G14V, suggesting that RhoA-Q63L is likely a better activating mutant [30]. For this reason, analogous glutamine to leucine mutants of RhoB and RhoC should be tested for interaction with Ipcef, although it is unlikely that such mutants will bind since the G14V mutants showed no trace of this ability.

That RhoA plays a role in this process while other notable GTPases in the Rho family appear to lack involvement adds to the body of evidence that Rho proteins have very specific functions in cellular events, despite their sequence homology. Even among the highly similar Rho subfamily drastic differences exist in their functions, which relates to their impacts for disease on an organism scale. Overexpression of RhoA and RhoC are often found correlated with cancers [4], while down regulation of RhoB has been shown to promote migration and invasiveness [28]. It is also likely that post translational modification influences the functions of Rho proteins. For instance, even for just one GTPase, RhoB, different prenylation modifications affect its activity in Ras transformation [29].

Another future aim will be to determine if the binding of RhoA to Ipcef is GTP dependent, as it was shown to be for CNK1 [24]. Using wild type RhoA as template, we created a T19N dominant negative mutant by site directed mutagenesis. We are still in the process of experimenting with this construct to obtain conclusive results. Once this work is completed it will help further define the binding characteristics between RhoA and CNK3.

Arf6 has been shown to be active at the leading edge of migrating cells [27], but the signals establishing this polarity remain unknown. RhoA has functions at the rear of the cell where it regulates tail contraction [1]. Since RhoA can interact with the Arf6 pathway by means

of Ipcef, we can propose a model for organizing cellular Arf6-GTP. RhoA could prevent signaling through Ipcef at the rear of the cell, thus limiting Arf6 activation to the leading edge. We are testing whether RhoA can decrease Arf6 activation *in vivo* after HGF exposure. As shown here, our preliminary results favor the notion that RhoA does indeed prevent Arf6 activation, but this observation must be confirmed.

The experiments are still inconclusive because GGA3 pulldowns require extensive optimization. Cells must be plated so that they are in islands during the addition of HGF. They must be able to grow and spread during their response to the growth factor, but not so much that the islands lose their free edges. Once the cells contact their neighbors, spreading signals will be shut off, resulting in Arf6 inactivation. When plating cells, a balance between area for growth and enough cells for detectable Arf6 signal has to be achieved, which is complicated by the effects of transfection and protein overexpression. A major focus of future work will be on developing reproducible conditions to achieve statistically significant results.

Another future aim is to visualize the spatial organization of Arf6-GTP in response to RhoA overexpression. A Fluorescence Resonance Energy Transfer (FRET) assay is being developed for this purpose. We expect that overexpressing constitutively active RhoA will decrease the area of Arf6 activation appearing at the leading edge, while a dominant negative RhoA mutant will fail to inhibit Arf6-GTP and lead to a broader range of activation.

We also investigated one potential direct molecular consequence of RhoA binding to Ipcef. With the knowledge that Ipcef binds cytohesin 2 and enhances its ability to activate Arf6 [19], we speculated that RhoA-GTP may prevent the binding of Ipcef to cytohesin 2 as a mechanism for inhibiting Arf6 activation. This model does not appear to be the case, though, since co-immunoprecipitation experiments demonstrated that Ipcef has the ability to bind RhoA-

GTP and cytohesin 2/ARNO simultaneously in the cell. This observation suggests RhoA-GTP inhibits this pathway by an alternative mechanism. To confirm that the data is consistent with the actual cellular CNK3 protein, this experiment should be repeated once a full length transcript is cloned.

If CNK3 can also bind RhoA-GTP and cytohesin 2 together, that poses an area for future research. If RhoA does in fact inhibit Arf6 activation, what is the mechanism through which it influences the signaling pathway? A few possibilities exist. RhoA binding could alter the structure of the CNK complex and prevent either the response of cytohesin 2 to upstream signals or the GEF ability of cytohesin 2 for activating Arf6. Alternatively, RhoA could induce the recruitment of other proteins that modify the structure or activity of the complex, or lead to the sequestration of the signaling complex in a different area of the cell.

This thesis provides evidence for a new mechanism by which RhoA can regulate cellular motility through influencing the actin cytoskeleton. Much work has been done on Rho induced cytoskeletal changes through the effectors mDia and ROCK, but here we explored a model where Rho organizes actin by regulating the activation of another GTPase. This research also opens up more questions. If RhoA is inhibiting Arf6 activation at the rear of the cell, then what is organizing the spatiotemporal dynamics of RhoA activation? Also, RhoA has recently been shown to be active at the tip of membrane protrusions [6], so future work should investigate whether RhoA is simultaneously regulating two separate processes. Determining the exact mechanism through which RhoA is influencing the CNK3 organized signaling complex will be another area for research.

Involved in normal functions and disease, epithelial cell migration is an intricate biological process requiring precise spatial organization and timing. Defining the regulation of

HGF induced Arf6 activation will uncover a new pathway through which motility occurs and illustrate the integral role of GTPases in regulating migration. By learning about the methods cells utilize to direct movement, we will better understand tissue morphogenesis, wound healing, and cancer metastasis. The exciting discoveries of basic science will also put us in better position to develop novel therapeutics targeting these processes in invasive cancers.

Materials and Methods

Cells

Mammalian: HeLa maintained in DMEM, 10% FBS, 1% PSF, 1% l-glutamine at 37˚ C in 5% CO_2 incubator. MDCK-T23 maintained in DMEM, 10% FBS, 1% PSF in at 37˚ in 5% CO_2 incubator.

E. coli: Commercial XL1-Blue, DH5-alpha, DH10-beta used for transformation and amplification of DNA constructs.

DNA constructs

pCB7 empty plasmid, pRK5-myc-RhoA-wt, pRK5-myc-RhoA-Q63L, pRK5-myc-Rac-Q61L, pRK5-myc-Cdc42-Q61L, pCDNA3-HA-Ipcef, and pCB7-myc-ARNO from Dr. Lorraine Santy. pCDNA3.1-HA-RhoB-G14V and pCDNA3.1-HA-RhoC-G14V purchased from Missouri University of Science and Technology and subcloned into pRK5-myc. pRK5-myc-RhoA-G14V and pRK5-myc-RhoA-T19N were generated by site-directed mutagenesis according to the QuikChange protocol (Stratagene).

Antibodies

Primary: Mouse anti-myc 9E10, rabbit anti-HA, and mouse anti-HA purchased from Covance. Rabbit anti-Arf6 is a gift from Dr. James Casanova.

Secondary: Goat anti-mouse and goat anti-rabbit both coupled to horseradish peroxidase purchased from Invitrogen.

Immunoprecipitation assay

8x10E5 HeLa cells were plated onto 6 cm plates. 24 hours later the cells were transfected with Lipofectamine 2000 reagent (Invitrogen) in serum/antibiotic free DMEM. 10% FBS was added to the media 4-5 hrs later. The cells were lysed in 1 ml of lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1x Triton, 10 mM NaF, 1 mM NaVO4, 10 mM NaPyrophosphate, 1:1000 protease inhibitors, 1:1000 DTT) 24 hrs after the transfection. Cell debris was pelleted by the addition of 30 ul Sepharose CL-4B (Fluka) followed by centrifugation at 13,000 rpm for 5 min at 4° C. Some of the supernatant (25 ul) was saved for analysis and the rest was incubated for 1 hr at 4° C with 1 ul rabbit anti-HA and then for at least 2 hr at 4° C with 25 ul protein A-sepharose beads. The immunocomplexes were washed 3 times with 1 ml lysis buffer. Samples from the whole cell extract and immunoprecipitation were run on a SDS-PAGE gel, transferred to nitrocellulose or PVDF, and blotted with mouse anti-HA or mouse anti-myc.

GGA3 Pulldown assay

6x10E5 MDCK-T23 cells were reverse transfected with either empty pCB7 plasmid (4 sets) or pRK5-myc-RhoA-Q63L (4 sets) using Lipofectamine LTX (Invitrogen) and plated into 35 mm well plates containing antibiotic free DMEM. After the cells settled onto the plates (about 3-4 hrs), the media was changed to DMEM plus 1% FBS to serum starve the cells overnight. The next morning HGF (100 ng/ml) was added to 2 wells containing mock transfected cells and 2 wells containing RhoA transfected cells. After 6 hours, the cells were lysed in 0.65 ml of pulldown buffer (200 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris pH 7.5, 1x Triton, 0.5% sodium deoxycholate, 0.1% SDS, 5% glycerol, 1:1000 protease inhibitors, 1:1000 DTT) and the duplicate plates combined into one solution. Cell debris was pelleted by the addition of 30 ul

Sepharose CL-4B (Fluka) followed by centrifugation at 13,000 rpm for 2 min at 4° C. Some of the supernatant (50 ul) was saved for analysis and the rest (500 ul) was added to GGA3-GST-sepharose fused beads containing at least 40 ng of GGA3 protein and incubated at 4° C rocking for 30 min. The beads were washed 3 times with 500 ul pulldown buffer. Samples from the whole cell extract and pulldown were run on a 13% SDS-PAGE gel, transferred to PVDF, and blotted with rabbit anti-Arf6.

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Experience

<u>Assistant under Dr. Lorraine Santy</u> — Penn State, University Park
Undergraduate Researcher — Sept. 2008 – Present

- Conducted immunoprecipitation experiments to assess protein binding
- Analyzed protein influence with a pathway via transfection and pull-downs
- Created unique proteins through site-directed mutagenesis
- Purified proteins using bacterial over-expression and metal affinity resins

<u>Genetics Instructor</u> — Penn State, University Park
Teaching Assistant — Jan. 2009 – May 2009

- Led six review sessions over the course of a semester
- Hosted office hours before exams
- Generated and analyzed exam questions

<u>Vector Marketing</u> — Blue Bell, PA
Sales Representative — Jun. 2008 – Aug. 2008

- Sold over $15,000 of fine cutlery by performing demonstrations
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Activities

<u>The Penn State Biochemistry Society</u> — Penn State, University Park
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- Rebuilt the membership to offer networking within the major
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<u>Phi Beta Lambda, Professional Business Fraternity</u> — Penn State, University Park
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