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***Dictyostelium discoideum* SecG interprets cAMP mediated chemotactic signals to influence actin organization**

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Abstract

Tight control of actin cytoskeletal dynamics is essential for proper cell function and survival. ARNO, a mammalian guanine nucleotide exchange factor for Arf, has been implicated in actin cytoskeletal regulation but its exact role is still unknown. To explore the role of ARNO in this regulation as well as in actin-mediated processes, the *Dictyostelium discoideum* homolog, SecG, was examined. SecG peaks during aggregation and mound formation. The overexpression of SecG arrests development at the mound stage. SecG overexpressing (SecG OE) cells fail to stream during aggregation. Although *carA* is expressed, SecG OE cells do not chemotax toward cAMP, indicating SecG is involved in the cellular response to cAMP. This chemotactic defect is specific to cAMP-directed chemotaxis, as SecG OE cells chemotax to folate without impairment and exhibit normal cell motility. The chemotactic defects of the SecG mutants may be due to an impaired cAMP response as evidenced by altered cell polarity and F-actin polymerization after cAMP stimulation. Cells overexpressing SecG have increased filopodia compared to wild type cells, implying that excess SecG causes abnormal organization of F-actin. The general function of the cytoskeleton, however, is not disrupted as the SecG OE cells exhibit proper cell-substrate adhesion. Taken together, the results suggest proper SecG levels are needed for appropriate response to cAMP signaling in order to coordinate F-actin organization during development.

Keywords

actin; ARNO; cAMP chemotaxis; *Dictyostelium*; SecG

INTRODUCTION

The actin cytoskeleton provides mechanical strength and morphology for the cell by serving as a mediator between the extracellular matrix and the internal cellular machinery, enabling a cell to appropriately respond to external cues. By reorganizing in response to extracellular and intracellular signaling, the actin cytoskeleton plays an integral role in a variety of cellular processes including motility [Wittmann and Waterman-Storer, 2001], adhesion [Le Clainche and Carlier, 2008], cytokinesis [Nanninga, 2001], and endocytosis [Schafer, 2002]. Given its extensive and complex role, tight control of the actin cytoskeleton is essential. Accordingly, many proteins are involved in its regulation.

One such regulator is ARNO (Arf nucleotide binding-site opener), a member of the ARNO/cytohesin family of mammalian guanine nucleotide exchange factors (GEFs). GEFs catalyze the exchange of GDP for GTP, activating their target protein. Also known as cytohesin-2, ARNO serves as a GEF for Arf proteins, small GTP-binding proteins of the Ras superfamily

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commonly involved in membrane trafficking [Nie et al., 2003]. ARNO has been implicated in actin cytoskeletal rearrangements [Frank et al., 1998a; Frank et al., 1998b], as well as the actin-based processes of cell motility [Turner and Brown, 2001], endocytosis [Shmuel et al., 2006], and exocytosis [Vitale et al., 2002]. In epithelial cells, the overexpression of ARNO results in the flattening of periphery cells and formation of large lamellipodia, as well as the stimulation of cell motility and the separation of periphery cells from their neighbors [Santy and Casanova, 2001]. ARNO has also been shown to mediate neurite outgrowth and branching [Hernandez-Deviez et al., 2004; Yamauchi et al., 2009], processes dependent upon cytoskeletal coordination. Given the wide-range involvement of the actin cytoskeleton in cellular function, a detailed understanding of ARNO's role in actin-based processes can bring new insights about key players in the upstream regulation of the actin cytoskeleton and the mechanisms behind actin-dependent processes.

ARNO is characterized by an N-terminal coiled-coiled domain, a central catalytic Sec7 domain, and a C-terminal pleckstrin homology (PH) domain [Frank et al., 1998a]. The coiled-coil domain mediates homodimerization and has been implicated in the localization of ARNO to the plasma membrane in polarized epithelial cells [Shmuel et al., 2006]. The Sec7 domain, named for its homology to yeast protein Sec7, catalyzes the exchange activity of ARNO [Chardin et al., 1996]. The neighboring PH domain positively regulates this exchange activity through its interaction with inositol phospholipids [Chardin et al., 1996].

Like other cytohesin family members, ARNO is promiscuous in its Arf activity. *In vitro* studies suggest that ARNO catalyzes the exchange of GDP for GTP to activate both Arf1 and Arf6 [Chardin et al., 1996; Frank et al., 1998a]. However, due to its common localization with Arf6 on the plasma membrane, ARNO has been suggested to serve as a GEF for Arf6 [Frank et al., 1998a]. The precise *in vivo* substrate for ARNO, however, remains controversial [Cohen et al., 2007], indicating that much remains to be understood concerning the complex relationship between Arfs and ARNO.

Dictyostelium discoideum is an excellent model for investigating the interactions between Arfs and ARNO as many mammalian signal transduction pathways are conserved in this organism. Signaling events involving Ras superfamily proteins participate in actin-related processes in *D. discoideum*, including a variety of GTPases, GEFs, and GAPs [Charest and Firtel, 2006; Lim et al., 2002; Vlahou and Rivero, 2006]. The *D. discoideum* genome contains a total of sixteen Arf, Arf-related, and Arf-like proteins. Components of the Arf signaling cascade appear to be conserved in this organism [Mondal et al., 2007]. The *secG* gene (Dictybase ID DDB091439) residing on chromosome 5, however, is the only gene homologous to mammalian ARNO. Like mammalian ARNO, the 986 amino acid SecG protein has a catalytic Sec7 domain and a C-terminal PH domain [Shina et al., 2010]. SecG also retains functional similarity to ARNO, as SecG has also been linked to the actin-dependent processes of cell-substrate adhesion and motility [Shina et al., 2010].

Dictyostelium discoideum are single celled amoeba during vegetative growth, but enter into multi-cellular development upon starvation (for review see [Mahadeo and Parent, 2006; McMains et al., 2008; Urushihara, 2009]). cAMP is released by starving cells and triggers chemotaxis, leading to cell aggregation. A mound develops through a complex process involving chemotaxis, cell differentiation, cell sorting, and adhesion, processes rooted in effective organization of the actin cytoskeleton [Kessin, 2001]. During mound formation, the uniform population of aggregated cells begins to differentiate into prespore and prestalk cell types [Araki et al., 1997; Huang and Pears, 1999; Weijer, 2004]. In addition to its role in cell aggregation, cAMP chemotaxis also contributes to mound formation through the process of cell sorting, the spatial organization of different cell types [Savill and Hogeweg, 1997; Vasiev and Weijer, 1999]. As prestalk cells are best able to respond to cAMP and

exhibit greater chemotactic motility than prespore cells, prestalk cells migrate toward the mound center and then sort to the top of the mound, forming the organizing center known as the tip [Vasiev and Weijer, 1999] [Mee et al., 1986]. The cells in the organizing tip coordinate an effective and synchronized response to cAMP to facilitate development beyond the mound stage [Dormann et al., 2000; Weijer, 2004]. The prespore cells are less responsive to cAMP and therefore lag behind the prestalk cells. Development culminates in the formation of a fruiting body, consisting of dead stalk cells supporting live spore cells on top.

In this work, we demonstrate a role for SecG, the *Dictyostelium discoideum* homolog of ARNO, in cAMP mediated actin organization. We show that SecG, by integrating cAMP signaling with actin cytoskeletal reorganization, is involved in *D. discoideum* development.

RESULTS

SecG has peak production during development

Mammalian ARNO is known to play a role in various actin dependent processes such as motility, as well as in actin cytoskeletal organization. *Dictyostelium discoideum* heavily relies on actin mediated processes throughout its development. To determine if the *D. discoideum* homolog of ARNO, known as SecG, also influences actin organization and actin dependent processes, the involvement of SecG in development was assessed. In *D. discoideum*, the expression pattern of a protein may potentially be correlated to its developmental function. Protein expression at a particular developmental stage may indicate this protein plays a role in the various processes occurring during that stage of development. To explore the protein expression pattern of SecG, cell lysates from each stage of development were harvested. A Western blot was then performed on the lysates utilizing antibodies raised against a C-terminal peptide of SecG in order to detect the presence or absence of SecG at each developmental stage. Consistent with the previously described mRNA pattern [Shina et al., 2010], Sec G is present throughout both vegetative and developmental stages (Figure 1A). SecG levels decrease as cells enter development and remain low during the initial stages of development. Maximum production of SecG occurs approximately 12 hours into development, when cells undergo aggregation to form mounds. As development progresses beyond the mound stage into the generation of fingers and early culminants at 16 and 20 hours, respectively, SecG levels again decrease. By 24 hours after the onset of development, when fruiting body formation is completed, SecG is barely detectable. SecG production is therefore developmentally regulated, with peak production of SecG occurring during the mound stage.

SecG is involved in *Dictyostelium discoideum* development at the mound stage

To gain a better understanding of SecG's role in development, the Tet-off system described by Blaauw et al [Blaauw et al., 2000] was utilized to overexpress *secG*. To confirm an increase in SecG production, the level of SecG in *secG* overexpressing (SecG OE) cell lysate was compared to SecG levels in lysate from wild type cells. To ensure equal loading of each sample, the intensity of each SecG band was normalized with actin by densitometry. As depicted in Figure 1B, Western blots probed for SecG indicate a 1.5 fold increase in SecG production in the SecG OE cells, as compared to endogenous levels in wild type cells. No difference in SecG expression was detected between Ax2 cells and cells transformed with empty vector, Tet7 cells. Attempts to suppress *secG* overexpression with the addition of tetracycline did not significantly reduce SecG levels.

As evidenced by its peak expression during mound formation, SecG likely plays a role during this stage of development. The influence of SecG on development was explored by

observing phenotypic differences between SecG OE cells and wild type cells. When seeded onto a bacterial lawn and allowed to develop, wild type cells fully complete the developmental cycle and form fruiting bodies. SecG OE cells, however, arrest at the mound stage of development, the stage after which SecG levels drastically decrease (Figure 2A). SecG OE cells do not progress beyond the mound stage after any additional time. Other transformants, with varying levels of *secG* over-expression, also displayed the same phenotypic defects (data not shown). Thus, proper regulation of *secG* expression appears to be important for development past the mound stage.

SecG mutants fail to stream

To better understand the developmental defects associated with SecG misexpression, the process of aggregation was examined in more detail. As starving cells become responsive to cAMP, they become polarized and chemotax toward the aggregation center in a head-to-tail manner known as streaming [Kriebel et al., 2003]. These streams of chemotaxing cells give rise to aggregates. Wild type and SecG OE cells were allowed to aggregate at low cell density in submerged culture. Wild type cells undergo streaming by 6 hours (Figure 2B). By 16 hours, wild type cells form aggregates at 56×10^3 cells/cm². In contrast, by 16 hours, SecG OE cells fail to stream and only form few aggregates, even at high cell concentrations. The failure of SecG OE cells to undergo cell streaming and complete development does not appear to be due to an inability to express developmental genes. RT-PCR reveals that the developmentally regulated gene, *carA*, is expressed in secGOE cells, albeit in a slightly delayed manner compared to wt cells (Figure 2C). *acaA*, another developmental marker, also followed the same expression pattern as the wild type cells, though the level of expression was lower in the SecG OE cells (data not shown). Taken together, the results suggest proper *secG* expression appears to be required for effective cell streaming and aggregation.

SecG is involved in cAMP chemotaxis

Cell streaming during aggregation is due in part to efficient chemotaxis to cAMP. Given the absence of cell streaming in the SecG mutants, it is possible these cells have flawed chemotaxis. To determine whether SecG plays a role in cAMP chemotaxis, under-agarose chemotaxis assays were performed. In these assays, the ability of cells to efficiently migrate out of a trough and under agarose toward a chemoattractant is determined. Wild type cells and SecG OE cells were starved for 5 hrs and then assessed for chemotaxis toward cAMP. Chemotaxis was defined by the speed, directionality, and chemotactic index of a cell. Consistent with previous findings [Hoeller and Kay, 2007; Myers et al., 2005], wild type cells crawl under the agarose and migrate toward increasing cAMP concentrations, with a speed of 10.20 ± 0.49 μ m/min, a directionality of 0.79 ± 0.05 , and a chemotactic index of 0.94 ± 0.02 . Unlike the wild type cells, SecG OE cells do not travel under the agarose toward higher concentrations of cAMP. Instead, they remain in the trough, suggesting that they are unable to chemotax toward cAMP. This argues for a role for SecG in chemotaxis toward cAMP.

This defect in cAMP chemotaxis could be due to a general defect in chemotaxis. To address this possibility, we examined the cells ability to chemotax to folate using the same assay. Vegetative *D. discoideum* cells chemotax toward folate as a means of tracking bacterial prey [Pan et al., 1975]. While folate chemotaxis and cAMP chemotaxis have been shown to utilize distinct cell-surface receptors and signaling pathways [Rifkin, 2001; Woznica and Knecht, 2006] the underlying mechanics of cell motility remain the same. Wild type cells chemotax to folate with a speed of 11.29 ± 0.71 μ m/min, a directionality of 0.61 ± 0.03 , and a chemotactic index of 0.78 ± 0.04 . Similar to wild type cells, SecG OE cells have a speed of 12.39 ± 1.09 μ m/min, a directionality of 0.64 ± 0.04 , and a chemotactic index of $0.71 \pm$

0.04 (Figure 3A). The values for the SecG OE cells are not statistically different ($p > 0.05$) from those of the wild type. This suggests that SecG is not involved in chemotaxis to folate, but is specifically involved in cAMP chemotaxis.

An inability to chemotax toward cAMP could be due to defective cell motility in starved cells or the inability to sense the presence of the chemoattractant. To distinguish between these two possibilities, the motility of wild type cells and SecG OE cells in the absence and presence of cAMP was determined. Wild type cells have been shown to exhibit a change in cellular speed in the presence of cAMP, a property termed chemokinesis [Chernyavsky et al., 2004; Tang et al., 2001]. Cells were starved for 6 hours in submerged cultures, then random motility was measured both before and after global stimulation with 5 μ M cAMP. In the absence of cAMP, wild type cells have a speed of 9.23 ± 0.31 μ m/min. SecG OE cells exhibit a similar speed of 9.59 ± 0.65 μ m/min, indicating that these cells do not have defective motility under starvation conditions (Figure 3B). However, in the presence of cAMP (Figure 3C), while wild type cells have drastically reduced their speed (5.77 ± 0.19 μ m/min), SecG OE cells have only slightly decreased cell speeds (7.92 ± 0.28 μ m/min). This suggests that the SecG OE cells respond differently to cAMP than wild type cells.

SecG plays a role in cell polarization in response to cAMP

In the presence of a cAMP gradient, chemotaxing cells become polarized along the gradient. The creation of cell polarity is critical for a cell to effectively move up a chemotactic gradient. As SecG OE cells do not chemotax toward cAMP, these cells may not properly polarize in response to a cAMP gradient. To address this, 5 hour starved wild type cells and SecG OE cells were placed in a cAMP gradient. After allowing time for the cells to sense the gradient, the cells were fixed and stained for F-actin. Wild type cells appear to effectively polarize along the cAMP gradient, while the SecG OE cells do not display the same polarity along the gradient (Figure 4A). Cell polarity was quantified by calculating the ratio of the minor axis to the major axis of individual cells. A value of 1 indicates a cell is a perfect circle and is not polarized. As a cell becomes more polarized, it becomes more elliptical, and therefore the value decreases. As seen in Figure 4B, wild type cells effectively polarize along the gradient, exhibiting an elongated phenotype with a circularity of 0.67 ± 0.02 . SecG OE cells, however, are significantly less polar than the wild type cells with a circularity of 0.81 ± 0.01 . This suggests a role for SecG in cell polarity in response to a cAMP gradient.

SecG is involved in F-actin polymerization in response to cAMP

As SecG OE cells fail to undergo cAMP chemotaxis and given that F-actin polymerization is involved in chemotaxis, these mutants may be deficient in F-actin polymerization. To explore this possibility, the ability of SecG OE cells to polymerize F-actin in response to cAMP was examined. Wild type cells and SecG OE cells were starved for 5 hours then stimulated with 1 μ M cAMP. Samples were then collected at various time points, immediately fixed, and their F-actin stained and measured.

As reported in the literature [Chen et al., 2003; Willard and Devreotes, 2006], cAMP stimulates a sharp 1.6-fold increase in F-actin polymerization in wild type cells after 5 seconds and a smaller, broader peak 60 seconds after stimulation (Figure 5A). SecG OE cells also display an increase in F-actin polymerization 5 seconds after cAMP stimulation, though the increase is smaller than that of wild type cells. F-actin polymerization levels quickly return to basal levels within 20 seconds after stimulation, sooner than in wild type cells. 30 seconds after stimulation, when wild type cells have basal levels of polymerization, SecG OE cells display a second peak of F-actin polymerization that persists until approximately 80 seconds after stimulation. As compared to wild type cells, the pattern of F-

actin polymerization in response to cAMP stimulation is distinctly altered in the SecG mutant cells.

To test if the variation in F-actin polymerization of the SecG OE cells is specific to cAMP stimulation, the ability of vegetative wild type cells and SecG OE cells to polymerize F-actin in response to folate was analyzed. In response to folate, F-actin polymerization can be visualized as a single peak approximately 5 seconds after stimulation [Tillinghast and Newell, 1987]. As depicted in Figure 5B, 5 seconds after stimulation with 50 μ M folate, wild type cells and SecG OE cells both display a sharp peak of F-actin polymerization between 1.3-fold and 1.6-fold that of basal levels. As the SecG OE cells are able to polymerize F-actin with similar timing as the wild type cells in response to folate, the defects observed in F-actin polymerization after cAMP stimulation are specific to cAMP.

Proper SecG expression is needed for actin organization

Given the defects in cAMP stimulated F-actin polymerization, SecG OE cells may have abnormalities in the organization of F-actin. To ascertain whether F-actin distribution is dependent upon SecG, F-actin was visualized in wild type and SecG OE cells using Rhodamine Phalloidin. As seen in Figure 6A, 6 hour starved wild type cells have F-actin distributed in filopodia and broad membrane protrusions. SecG OE cells, however, display an increased number of filopodia compared to wild type cells and have bright spots of cytosolic F-actin. As depicted in Figure 6B, the average mean intensity per cell of the SecG OE cells was $95\% \pm 9\%$ of the wild type mean intensity. As wild type cells and SecG OE cells have a similar mean intensity per cell, the overall amount of F-actin is not altered by the change in SecG levels. Taken together, the data suggest that the overexpression of SecG causes distinct changes in F-actin localization compared to wild type cells, without significantly changing the overall levels of polymerized actin.

Altered SecG expression does not impact cell adhesion

In addition to its involvement in chemotaxis [Swaney et al., 2010], the cytoskeleton plays a role in a multitude of eukaryotic cellular processes including adhesion [Le Clainche and Carlier, 2008]. To investigate whether the chemotactic defects of the SecG OE cells are indicative of an impairment in the general function of the cytoskeleton, the cell-substrate adhesion of these cells was assessed. Wild type and mutant cells were allowed to adhere to a glass surface and then gently agitated. The non-adhered cells in the supernatant were counted and subtracted from the total cell number as a measure of cell-substrate adhesion. As depicted in Figure 7, $87 \pm 2\%$ of wild type cells and $79 \pm 7\%$ of SecG OE cells adhere to the substratum. SecG OE cells do not differ in their ability to adhere to a substratum, as compared to wild type cells, suggesting general cytoskeletal function is not impacted.

DISCUSSION

The responsiveness of a population of individual, starving cells toward cAMP is essential for proper cell aggregation and multi-cellular organization during *Dictyostelium discoideum* development. In this light, the mound arrest of the SecG OE cells may be the result of a defective cAMP response. Booth et al [Booth et al., 2005] suggest SecG plays a role in cAMP chemotaxis based upon coordinate expression and regulation with key chemotaxis genes. Consistent with its expression pattern, SecG appears to be involved in the cAMP response during aggregation when peak production occurs. After the mound stage, when cells have initially differentiated and sorted according to cell-type, SecG levels decline and culmination of fruiting bodies ensues. This suggests SecG is required during aggregation and mound formation, but needs to be removed after the mound has formed. It is possible that the SecG OE cells arrest at the mound stage because SecG levels never decrease,

preventing progression to the next developmental stage. SecG null cells were reported to display a normal developmental phenotype [Shina et al., 2010]. It is possible there is partial redundant function between SecG and the protein product of Dictybase ID DDB0233617 which also contains a Sec7 domain and a PH domain. This gene is expressed 4 hours into development and, like *secG*, decreases in expression by 12 hours [Gregor Rot, 2010]. The similarity in functional domains may enable this protein to compensate for the absence of SecG in the null cells, preventing a complete view of SecG function.

Closer examination of the mound formation process with low density aggregation assays revealed SecG OE cells are unable to undergo cell streaming. This suggests that the SecG OE cells do not truly aggregate in response to chemoattractant signaling, but form mounds by adhering to neighboring cells at higher densities. The SecG OE cells, however, do enter the developmental phase as *carA*, a common developmental marker, is expressed 8 hours after the onset of starvation. Under agarose chemotaxis assays suggest the inability of the SecG OE cells to stream may be due to their failure to chemotax toward cAMP. This impairment in chemotaxis is likely due to a faulty cAMP signaling cascade as the SecG OE cells display cell motility comparable to wild type cells in the absence of cAMP and significantly altered cell speed after addition of cAMP. This defect is specific to cAMP mediated chemotaxis, as chemotaxis to folate is not impacted in SecG OE cells. Thus, there is not a defect in general chemotaxis, but rather a deficiency in cAMP relay. Manipulating SecG expression blocks the ability of a cell to properly respond to cAMP, thereby preventing chemotaxis and possibly streaming, implying that SecG is involved in the chemotactic response to cAMP. Cells lacking SecG were previously reported to undergo cAMP chemotaxis with reduced cell speed [Shina et al., 2010]. As the SecG OE cells have altered cell speed after global stimulation with cAMP, SecG may be involved in regulating cell speed during cAMP chemotaxis.

Directional cell movement is a multifaceted process involving the detection of the chemoattractant gradient, establishment of a leading edge, and cell polarization to generate forward cell movement [Arigoni et al., 2005; van Haastert and Devreotes, 2004]. As the SecG OE cells do not display appropriate cell speed or cell polarity in response to cAMP, these cells either do not sense the chemoattractant or cannot respond to the chemical signal. Such impairments are often due to deficiencies in F-actin polymerization. F-actin is polymerized in discrete regions along the chemotactic gradient, driving cell polarity and enabling pseudopod extension at the leading edge of a cell and therefore migration toward the chemoattractant [Arigoni et al., 2005; Kimmel and Firtel, 2004].

In response to cAMP, *D. discoideum* cells polymerize F-actin 5 seconds and 60 seconds after stimulation [Chen et al., 2003; Willard and Devreotes, 2006]. This second F-actin polymerization peak, associated with pseudopod extension, is thought to play a role in optimizing the chemotactic response as its disruption results in poor chemotaxis [Willard and Devreotes, 2006]. In SecG OE cells, F-actin is moderately polymerized 5 seconds after cAMP stimulation but polymerization quickly returns to basal level. Polymerization again increases 20 seconds after stimulation and remains at an elevated level until 80 seconds after stimulation. SecG may be involved in regulating the timing of F-actin polymerization in response to cAMP as SecG over production leads to premature polymerization in that the second peak occurs 40 seconds earlier than in wild type cells. This early second peak may cause the observed polarity defects in these cells. As both the timing and intensity of the second F-actin polymerization peak in the SecG OE cells differ from that of wild type cells, SecG OE cells may have altered pseudopod formation, which can interfere with proper chemotaxis. The defects in F-actin polymerization of SecG OE cells are specific to cAMP as polymerization in response to folate follows the same timing as polymerization in wild type cells, that being a characteristic single peak at 5 seconds [Tillinghast and Newell, 1987].

While the intensity of this polymerization does differ from that of wild type cells, the SecG OE cells are not likely impacted as folate chemotaxis occurs without impairment. Taken together the results suggest SecG is important for a cell to appropriately sense and respond to cAMP in order to polarize, polymerize actin, and ultimately undergo chemotaxis. This function may be correlated to role of mammalian cytohesins in stimulating the reorganization of actin to generate membrane ruffling, leading to cell migration [Santy and Casanova, 2001].

As directed cell movement is heavily dependent upon the actin cytoskeleton, defects in F-actin organization can lead to impaired cell polarity and chemotaxis. The overproduction of SecG yields an increased number of filopodia compared to wild type cells and bright spots of cytosolic F-actin. As F-actin is localized to the cell periphery, particularly the leading edge, during chemotaxis, it is possible that the localization of F-actin to cytosolic puncta in the SecG OE cells may impede their chemotactic ability. In addition, the excessive filopodia present in these cells, coupled with altered F-actin polymerization, may impair effective movement up the chemotactic gradient. The overproduction of SecG specifically disrupts polarity and cAMP chemotaxis rather than general cytoskeletal function as the SecG mutant cells exhibit levels of cell-substrate adhesion comparable to those of wild type cells. This also demonstrates that the cAMP chemotaxis defect in the SecG OE cells is not due to cell-substrate adhesion. These findings are consistent with SecG being an upstream regulator of F-actin organization, like its mammalian homolog [Frank et al., 1998b].

A wide variety of proteins serve as upstream regulators of F-actin organization. Members of the Ras superfamily of small guanine nucleotide triphosphatases (GTPases), such as Ras, Rho, and Rac, organize the actin cytoskeleton through various signal transduction pathways in response to activation of cell surface receptors and guanine nucleotide exchange factors (GEFs) [Hall, 1998; Schmidt and Hall, 1998]. The overexpression of *rasC* leads to abnormal F-actin organization and excessive cellular protrusions, much like the SecG OE cells [Seastone et al., 1998]. Several *D. discoideum* Ras and Rac GEFs have been associated with actin cytoskeletal organization. SecG likely serves as a GEF for an undetermined Arf family protein to coordinate chemotactic signals and cytoskeletal rearrangements. Like its mammalian homolog, SecG appears to regulate organization of the actin cytoskeleton. Given these similarities, a new avenue for exploring many of the unknown signaling and regulatory events surrounding ARNO can be examined using *D. discoideum* SecG as a model. For instance, ARNO has been shown to activate PLD activity in epithelial cells [Santy and Casanova, 2001], yet the precise mechanism of action remains to be elucidated. This question can readily be addressed by decoding the signaling pathway of SecG and the PLD homolog, PldB. Given the relative ease of genetically modifying *D. discoideum*, the SecG domains involved in this signaling can be mapped by developing various mutations in the *secG* gene.

Given its extensive role in a cell, a clear and thorough understanding of actin cytoskeletal regulation and organization is fundamental to demystifying the complex network of signal transduction pathways that coordinate physiological processes. This work highlights the role of SecG as an integral player in relaying chemotactic signals to coordinate the actin cytoskeleton. An understanding of SecG can shed light on the signaling events that culminate in *Dictyostelium discoideum* development as well as those involved in mammalian systems.

MATERIALS AND METHODS

Cell Culture and development

Dictyostelium discoideum wild type Ax2 cells were grown axenically in HL5 medium at 22°C in shaking culture [Sussman, 1987]. Ax2 cells carrying the endogeneous MB35 plasmid (Tet7) were grown in HL5 supplemented with 5 µg/ml G418. SecG overexpressing cells (SecG OE) cells were grown in HL5 supplemented with 20 µg/ml G418 and 10 µg/ml Blasticidin S. For development, 1×10^7 cells in mid-log phase (2.5×10^6 cells/ml) were washed with PBM (20 mM KH₂PO₄, 0.01 mM CaCl₂, 1 mM MgCl₂, pH 6.1 with KOH), plated onto filter pads, and then incubated at 22°C [Jain et al., 1992]. For development on agar, wild type cells were grown on a lawn of *Klebsiella aerogenes* while mutant strains were grown on a lawn of G418 resistant *Escherichia coli*.

Generation of SecG OE cell lines

The tetracycline-inducible system described by Blaauw et al [Blaauw et al., 2000] was utilized to overproduce SecG in *D. discoideum*. The full length *secG* gene was amplified by PCR with primers containing the restriction enzyme site for SphI at the 5' end (GCGCATGCATGGGATCAACATCAAATTCA AC) and AatII at the 3' end (CGGACGTCCTAAGATAATGCAGAAGTTTGTG) using the Roche Expand High Fidelity PCR System (Indianapolis, IN), with the following cycling conditions: 94°C for 45 sec, 58°C for 1 min, and 72°C for 2 min for 10 cycles followed by 94°C for 45 sec, 62°C for 1 min, and 72°C for 2min and 5 sec for 25 cycles. The generated PCR product was then cloned into the MB38 vector using the SphI and AatII restriction sites. The SecG OE cells were generated by transforming the MB38 plasmid carrying the full length *secG* gene into Tet7 cells. Transformants were selected on GYP plates containing 20µg/ml G418 and 10µg/ml Blasticidin S.

Western Blot Analysis

The synthetic peptide GCEQLLKRKAETIRGRGKVS, corresponding to amino acids 901 to 918 in the C-terminal portion of SecG, was utilized to immunize a rabbit at Biosynthesis, Inc (Lewisville, Tx). Serum was collected 8 weeks after a second boost and affinity purified using the above peptide. Cells were collected and boiled for 3 min in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, transferred onto Hybond-P membranes (Amersham Bioscience, Piscataway, NJ), immunoblotted with α-SecG antibody, and visualized with enhanced chemiluminescent substrate for HRP detection (PIERCE, Rockford, IL).

Low Cell Density Assay

Cells were starved at 22°C for 16 hrs in submerged monolayer culture at densities of 224×10^3 , 112×10^3 , 56×10^3 , 28×10^3 , 14×10^3 , and 7×10^3 cells/cm² in PBM. Images were taken after 6 hours with an inverted Nikon TE 200 Eclipse microscope using Metamorph Image System (Molecular Devices, Downingtown, PA) viewed with a 10x objective.

Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA was isolated from cells starved on filter pads at the times indicated using the Rneasy mini kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol, and used as template to create cDNA using GoScript Reverse Transcriptase (Promega, Madison, WI) and oligo dT primer (Promega, Madison WI). PCR amplification was performed using GoTaq Green Master Mix (Promega, Madison, WI). The upstream primer for *carA* was 5'-GGATTGGTGTAAGTTTCACTGG-3', and the downstream primer was 5'-CCATATCGGAACACTACATTGCAC-3'. The upstream primer for IG7 was 5'-

TTACATTTATTAGACCCGAAACCAAGCG-3', and the downstream primer was 5'-TTCCCTTTAGACCTATGGACCTTAGCG-3'. The PCR products were separated on a 2% agarose gel and visualized with ethidium bromide. Duplicate reactions were performed without reverse transcriptase to control for DNA contamination in the RNA preparation.

Chemotaxis Assay

Under agarose chemotaxis assays were performed as described by Woznica and Knecht [Woznica and Knecht, 2006], with some adjustments. For cAMP chemotaxis, 1×10^7 cells were starved on filter pads for 5 hrs. Cells were collected and resuspended in 1 ml PBM. A 100 μ l sample of cells was used in the assay. For folate chemotaxis, vegetative cells were collected and resuspended to 1×10^6 cells/ml and 100 μ l used in the assay. Images of cells migrating under agarose toward 10 μ M cAMP or 1 mM folate were taken every 30 sec for 20 min on an inverted Nikon TE 200 Eclipse microscope using Metamorph Image System (Molecular Devices, Downingtown, PA) viewed with a 10x objective. Cells were tracked with Image J software. Directionality is defined as (absolute distance traveled)/(total path length), where a maximum value of 1 represents a straight path without deviations. The chemotactic index is determined by calculating the $\cos \theta$, where θ represents the angle of deviation between a straight line up the chemotactic gradient and the net path of a cell. A value of 1 is equivalent to a straight path up the chemotactic gradient.

Cell Motility Assay

1×10^6 cells were collected, washed with PBM, and resuspended in 10 ml PBM. 200 μ l of cells were placed into a 24 well plate with 200 μ l PBM. Cells were starved for 6 hrs. cAMP was then added to the cells for a 5 μ M final concentration. Images of cells in the absence and presence of cAMP were taken every 30 sec for 20 min on an inverted Nikon TE 200 Eclipse microscope using Metamorph Image System (Molecular Devices, Downingtown, PA) viewed with a 10x objective. Cells were tracked with Image J software.

Cell Polarization Assay

A cAMP gradient was established as described by Woznica and Knecht [Woznica and Knecht, 2006], with some modifications. Briefly, PBM + 0.75% agarose was poured into a 4-well Millicell EZ slide (Millipore, Cork, Ireland). After solidifying, the agarose was removed from half of each well. Using a pipet tip, a small well was made 5 mm from the edge of the agarose. The well was then filled with 10 μ M cAMP + 20 mg / ml FITC-dextran (Sigma, St. Louis, MO) and a gradient was allowed to develop. 5 hr starved cells were then placed onto the slide for approximately 1×10^4 cells/ cm^2 . Establishment of the cAMP gradient was confirmed by measuring the FITC-dextran gradient by fluorescence on an inverted Nikon TE 200 Eclipse microscope using Metamorph Image System (Molecular Devices, Downingtown, PA) viewed with a 10x objective. Cells were allowed to orient for 1 hour, and then fixed and stained for actin as described below. Images were taken on a Leica confocal microscope. Cell circularity was measured utilizing Image J software.

F-Actin Polymerization Assay

For F-actin polymerization in response to folate, 5×10^7 cells in the vegetative state were washed with PBM and resuspended in 10 ml PBM for 5×10^6 cells/ml. After shaking for 30 min, cells were washed and resuspended in 1 ml PBM. Cells were stimulated with 50 μ M folate. 100 μ l samples were collected after 0, 5, 10, 20, and 60 seconds and placed into 500 μ l actin fixative buffer (10 mM PIPES, pH 6.8, 20 mM K_2HPO_4 , 5 mM EGTA, 0.5% Triton X, 3.7% formaldehyde, 0.25 mM AlexaFluor Phalloidin 488). For F-actin polymerization in response to cAMP, 1×10^7 cells were starved for 5 hrs on filter pads. The cells were then collected, washed with PBM, and resuspended to 2 ml PBM. Cells were stimulated with 1

μM cAMP. 100 μl samples were collected after 0, 5, 10, 20, 30, 40, 50, 60, 80, 100, and 150 seconds and placed into 500 μl actin fixative buffer.

The samples were incubated in the dark for 1 hr and then centrifuged at 1300 rpm for 10 min at 4 °C. The supernatant was removed and the pellet resuspended in 1 ml methanol. The samples were incubated at 4 °C on a rotatory shaker overnight. 100 μl of each sample was placed onto a plate reader for measurement of fluorescence.

Actin Staining

Cells were washed twice with PBM and starved on an 8 well chambered slide for 6 hrs. Cells were fixed (3.7% formaldehyde in PBS for 5 min), washed with PBS, permeabilized (0.5% Triton X-100 in PBS), and washed with PBS. Cells were incubated with 100 $\mu\text{g/ml}$ RNaseA for 1 hr. After a PBS wash, cells were stained with 8 U/ml Rhodamine Phalloidin (Invitrogen Eugene, OR) for 1hr in the dark. After a PBS wash, cells were incubated with 20 μM To-Pro (Invitrogen Eugene, OR) for 15 min. Slow Fade Gold (Invitrogen Eugene, OR) mounting solution was added prior to placing a coverslip onto the slide.

Cell-Substrate Adhesion Assay

Cell-substrate adhesion was performed as described by Chen and Katz [Chen and Katz, 2000] with slight modifications. A suspension of 1×10^6 cells in 1 ml of PBM was placed in a 125 ml glass culture flask and put on a gyratory shaker for 10 min at 120 rpm. The cells were allowed to adhere to the glass substrate for 2 hrs. The flask was then gently agitated for 3 min at 60 rpm. The supernatant was collected and the number of cells present was counted with a hemacytometer.

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Figure 1.

SecG protein production in wild type and mutant cells. **(A)** 1×10^7 wild type cells were collected at different developmental time points. After separation by SDS-PAGE, proteins were identified by Western blotting with anti-SecG antibodies. **(B)** 1×10^7 wild type (WT) and SecG overexpressing (OE) cells were collected, and their proteins separated by SDS-PAGE. SecG was identified by Western blotting with anti-SecG antibodies. To ensure equal loading, actin was detected with anti-actin antibodies and quantified by densitometry.

Figure 2.

SecG OE cells express *carA*, but do not undergo cell streaming during development. **(A)** Wild type (WT) and SecG overexpressing (SecG OE) cells were seeded onto a bacterial lawn over agar and allowed to develop. Bar, 0.25 mm. **(B)** Wild type and SecG OE cells were starved for 6 hrs under PBM at 56×10^3 cells/cm² and 224×10^3 cells/cm², respectively. Bar, 1 mm. **(C)** cDNA using oligo dT primer was created from RNA isolated from vegetative and starved wild type and SecG OE cells, and RT-PCR performed with primers for *carA*. Simultaneous reactions were performed with primers for the IG7 gene to ensure that equal amounts of total RNA and cDNA were used in each of the samples.

Figure 3.

SecG OE cells undergo chemotaxis to folate, but have altered motility in the presence of cAMP. (A) Time-lapse images of vegetative cells migrating under agarose toward 1 mM folate were taken. Individual cells were tracked with ImageJ software. Directionality is defined as net distance / total path. The chemotactic index is calculated as the $\cos \theta$, where θ is the angle of deviation between a direct line up the chemical gradient and the net path of a cell. Values represent the mean of >5 experiments $\pm$ SEM. A minimum of 25 cells were measured for each experiment. Cells were starved for 6 hrs under PBM. Time-lapse images were taken before (B) and after (C) global stimulation with 5 μ M cAMP. Individual cell were tracked with ImageJ software. Values represent the mean of >3 experiments $\pm$ SEM. A minimum of 25 cells were measured for each experiment. *, $P < 0.01$ compared with wild type control (Student's t test).

A.

B.

Figure 4.

SecG mutants have polarity defects in cAMP gradient. (A) Cells were starved for 5 hrs then placed in a cAMP gradient with a maximum concentration of 10 μ M. Cells were fixed and stained with Rhodamine-Phalloidin to visualize F-actin organization. Bar, 15 μ m. (B) Cell polarity was quantified with Image J software and measured as the ratio of a cell's minor axis to its major axis. Values represent the mean of over 90 cells $\pm$ SEM over the course of three trials. *, $P < 0.01$ compared with wild type control (Student's t test).

Figure 5.

SecG mutants polymerize F-actin differently than wild type cells. F-actin polymerization was quantified by measuring the level of Alexa fluor 488 Phalloidin in samples of wild type and SecG OE cells taken at various time points after stimulation by (A) 1 μ M cAMP or (B) 50 μ M folate. Values represent the mean of >4 experiments $\pm$ SEM.

Figure 6.

Actin organization is altered in SecG mutant cells. **(A)** Cells were starved for 6 hrs, fixed, and stained with Rhodamine-Phalloidin to visualize F-actin organization. White arrows indicate sites of membrane protrusions. Gray arrows indicate filopodia. Bar, 10 μ m. **(B)** The mean intensity of Rhodamine-Phalloidin per cell was quantified with Image J software. Values represent the mean of at least 15 representative cells $\pm$ SEM over the course of three trials.

Figure 7.

SecG mutant cells and wild type cells have comparable cell-substrate adhesion. 1×10^6 cells were allowed to adhere to a glass surface for 2 hrs. After gentle agitation, the number of cells in the supernatant was counted and the percent of adhered cells calculated.