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RAC1 regulates actin arrays during polarity establishment in the brown alga, *Silvetia compressa*

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ABSTRACT

Multicellular development has evolved independently on numerous occasions and there is great interest in the developmental mechanisms utilized by each of the divergent lineages. Fucoid algae, in the stramenopile lineage (distinct from metazoans, fungi and green plants) have long been used as a model for early development based on unique life cycle characteristics. The initially symmetric fucoid zygote generates a developmental axis that determines not only the site of growth, but also the orientation of the first cell division, whose products have distinct developmental fates. Establishment and maintenance of this growth axis is dependent on formation of a filamentous actin array that directs vesicular movement, depositing new membrane and wall material for development of the rhizoid. What is not well known, is how formation and placement of the actin array is regulated in fucoid algae. A candidate for this function is Rac1, a small GTPase of the highly conserved Rho family, which has been implicated in controlling the formation of actin arrays in diverse eukaryotes. We demonstrate that Rac1 is not only present during formation of the filamentous actin array, but that its localization overlaps with the array in polarizing zygotes. Pharmacologically inhibiting Rac1 activity was shown to impede formation and maintenance of the actin array, and ultimately polar growth. Evidence is provided that a requirement of Rac1 function is its ability to associate with membranes via a post-translationally added lipid tail. Taken together, the data indicate that Rac1 is a necessary participant in establishment of the growth pole, presumably by regulating the placement and formation of the actin array. A role for Rac1 and related proteins in regulating actin is shared by animals, plants, fungi and with this work, brown algae, thus a conserved mechanism for generating polarity is in operation in unique eukaryotic lineages.

Introduction

The mechanisms regulating development have profound evolutionary significance given that complex multicellular development has independently evolved numerous times. The extent to which different lineages use the same basic mechanisms or have employed unique strategies to drive their muticellularity is less clear. Development, being a coordinated process of cell division and differentiation, results in forming tissues and organs that are arranged in patterns that facilitate specific functions and give a polarized, consistent shape to the adult organism. The primary growth axis of multicellular organisms often forms while still a single cell, either through inherited maternal influence on an egg, or after fertilization with external influences on the zygote. A classic example of maternally influenced polarity is *Drosophila melanogaster*, in which polarity determinants in the form of mRNAs and proteins like bicoid and hunchback are selectively localized during oogenesis (Huynh and Johnston, 2004). Alternatively, eggs of *Caenorhabditis elegans* are symmetric when formed, and establishment of polarity occurs with sperm entry, which marks the posterior portion of the organism (Goldstein and Hird, 1996).

The fucoid algae, a family of brown algae comprising the genera *Fucus* and *Silvetia*, have a strategy similar to *C. elegans* in that they produce eggs in which polarity has yet to be established. Apolar eggs are released from maternal tissues and begin to generate polarity after external fertilization, one of several features that have historically made the fucoid algae valuable models for the study of polarity establishment from its inception (Kropf, 1992). Sperm entry initially establishes polarity; yet in contrast to *C. elegans*, the fucoid developmental axis is labile and is usually re-oriented by environmental cues, like sunlight. Additionally, thousands of gametes can be procured within a very short time, thus a large population of synchronously developing zygotes can be obtained in the lab, and their polarity is easily manipulated by an artificial gradient of light. Moreover, because the developmental axis is labile for a period of several hours, experiments using a pharmacological approach in combination with cell biological techniques can elucidate the roles of specific proteins during

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polarity establishment. In spite of these tools, there is presently a greater understanding of the mechanisms regulating polarized growth in the plants, animals and fungi due to an enormous amount of prior work using those models; however, recent insights using the fucoid model are beginning to reveal the signaling requirements of cell polarization in the stramenopile lineage (Bisgrove, 2007; Hable and Hart, 2010).

In zygotes of *Silvetia compressa*, it is well documented that polarity establishment is dependent on a dynamic actin cytoskeleton (Quatrano, 1973; Hable and Kropf, 1998; Bisgrove and Kropf, 2001; Hable et al., 2003), and our interest is in how the actin cytoskeleton is regulated. In nature, sperm and eggs are released in calm tide pools and undergo rapid fertilization (Serrao et al., 1996). Within the cortex of an unfertilized egg are short, uniformly distributed strands of filamentous actin (F-actin) (Kropf et al., 1989). The first detectable break in symmetry, seen as early as 30 min after fertilization (AF), is the formation of a small patch of F-actin in the zygote cortex at the site of sperm entry, marking the rhizoid pole of a default growth axis (Hable and Kropf, 2000). The rhizoid pole is the site from which growth occurs several hours later.

Prior to rhizoid growth, within about 3 h of fertilization, zygotes adhere to the rocky substratum due to the uniform secretion of adhesive mucilage (Hable and Kropf, 1998), composed of phenolic compounds cross-linked to carbohydrate fibers (Vreeland et al., 1998). Once immobilized, the zygote assesses environmental cues and abandons the weak, sperm induced axis to form a new growth axis (Alessa and Kropf, 1999; Hable and Kropf, 2000) based primarily on unidirectional light of blue wavelengths (Jaffe, 1968). In the natural environment sunlight is likely the strongest cue that directs placement of this axis, however other cues have been identified, including gradients of chemicals, ions and temperature (Weisenseel, 1979) as well as unidentified influences from a near neighbor and bioluminescence from nearby algal thalli (Jaffe, 2005). In response to light, the sperm induced F-actin patch depolymerizes and a new F-actin patch forms at the shaded hemisphere, directing the position of rhizoid outgrowth (Alessa and Kropf, 1999; Hable et al., 2003).

At about 12 h AF, germination of the rhizoid begins, first noticed as a bulge in the shaded hemisphere of light-polarized zygotes that gives the zygote a pear shape. The rhizoid continues to develop by a process of tip growth that greatly extends its length relative to its width (Kropf, 1992), similar to that seen in root hairs and pollen tubes of plants (Cole and Fowler, 2006). Concurrent with fucoid rhizoid development is a reorganization of the F-actin patch into a cone-shaped array that spans from the nucleus to the sub-apical region of the rhizoid tip (Alessa and Kropf, 1999; Pu et al., 2000). The leading edge of this F-actin cone is maintained to the sub-apex of the developing rhizoid, with the primary function perhaps being to restrict delivery of vesicles to the rhizoid tip.

Not only are specific actin arrays associated with the rhizoid pole and tip, pharmacological studies either depolymerizing or stabilizing F-actin have shown that dynamic actin arrays are required for polarization (Quatrano, 1973; Hable and Kropf, 1998; Bisgrove and Kropf, 2001; Hable et al., 2003). Yet, little is known of how these actin arrays are regulated. There are several factors capable of nucleating actin arrays and Arp2, a subunit of the actin-nucleating Arp2/3 complex, has been identified in *S. compressa*. Additionally, colocalization of Arp2 with the F-actin patch and F-actin cone suggests the Arp2/3 complex has a role in nucleating these structures as it does in other eukaryotes (Hable and Kropf, 2005). The Arp2/3 complex, in turn, may be indirectly activated by the small GTPase, Rac1. Small GTPases of the Rho family (Rho, Rac and CDC42 in metazoans and fungi, and Rho of plants (ROPs)) stimulate actin polymerization by directly activating WASp/SCAR family proteins that themselves activate the Arp2/3 complex (Ridley, 2006). Within the brown algae, Rac1, but not Cdc42 or Rho, has been identified in the fully sequenced genome of *Ectocarpus siliculosus* (Cock et al., 2010) and Rac1 has been cloned from *Fucus distichus* (Fowler et al., 2004). Furthermore, the Rac1 pharmacological inhibitor NSC23766 (NSC) disrupts developmental events in *S. compressa* that are dependent on a dynamic actin cytoskeleton, and alters localization of F-actin and Arp2 within the rhizoid (Hable et al., 2008). This evidence suggests that Rac1 may regulate the actin cytoskeleton during fucoid algal development.

Here, we have examined the expression of Rac1 protein in *S. compressa*, showing that spatial and temporal expression of this protein supports a role in regulating actin during formation of the growth axis. Additionally, we show that Rac1 activity and membrane localization are required for the formation and maintenance of actin arrays, and for actin dependent processes, including rhizoid formation.

Materials and methods

For all experiments, a minimum of three replicates were carried out. In all cases the same trend was observed for each replicate. Figures show one replicate, a representative result for each experiment.

Algal cultures

Sexually mature *Silvetia compressa* receptacles were collected north of Santa Cruz, CA and were shipped overnight on ice. Upon delivery, they were blotted dry with paper towels and stored at 4 °C in darkness for up to 4 weeks. Receptacles were potentiated for gamete release by placing them in artificial seawater (ASW: 0.45 M NaCl, 10 mM KCl, 9 mM $CaCl_2 \cdot 2H_2O$, 30 mM $MgCl_2 \cdot 6H_2O$, 16 mM $MgSO_4$, 10 mM Tris base, 40 μg/ml chloramphenicol, buffered to pH 8.2 with HCl), under a full spectrum white light bank at 16 °C for between 6 h to overnight. After washing receptacles three times with ASW, gamete shedding was induced by placing them in darkness. Fertilization time was determined by taking the midpoint between the time receptacles were placed into darkness and the time zygotes were harvested. Since release of gametes required about 1 h, fertilization time was usually 30 min after placement in darkness also at 16 °C.

The zygotes were then washed three times by allowing them to settle to the bottom of a beaker, after which most of the ASW was removed with a pipette and replaced with fresh ASW. The zygotes were then plated onto Petri dishes typically containing coverslips secured to the bottom with modeling clay. All subsequent incubations occurred at 16 °C.

Production of polyclonal Rac1 antibody

The Rac1 antibody was produced by Covance Inc, Denver, PA. A Rac1-specific peptide was derived from a well-conserved 22 amino acid segment present in Rac1 from *Fucus distichus* (Fowler et al., 2004) and *Ectocarpus siliculosus* (*Ectocarpus* genome consortium; Cock et al., 2010). The peptide sequence (CILVGTKLDLRDDQDAIKRLAER, which includes an added N-terminal cysteine) was synthesized and injected into two New Zealand White rabbits. Periodic assessments of antibody development in crude sera were made by western blot analysis of protein extracted from *S. compressa*. From the total serum of the rabbit that exhibited the best immune response, antibodies were affinity purified using a column to which the antigen (the 22 amino acid peptide) was immobilized.