ORIGINAL ARTICLE

The Rac1 inhibitor, NSC23766, depolarizes adhesive secretion, endomembrane cycling, and tip growth in the fucoid alga, *Silvetia compressa*

Whitney E. Hable · Sriharshan Reddy · Lindsay Julien

Abstract Proper cell morphogenesis is dependent on the establishment and expression of cellular polarity. In the fucoid zygote, cell shape is critical for establishing the developmental pattern of the adult, and is achieved by guiding insertion of new membrane and wall to the rhizoid tip. Selection and growth of the appropriate tip site are accompanied by formation of dynamic actin arrays associated with the actin-nucleating Arp2/3 complex. In eukaryotes, a major pathway for activation of the Arp2/3 complex is via the Rho family GTPase, Rac1, which stimulates the Scar/WAVE complex. To determine whether Rac1 controls actin nucleation in *Silvetia compressa* (J. Agardh) E. Serrao, T. O. Cho, S. M. Boo et Brawley, we tested the effects of the Rac1-specific inhibitory compound, NSC23766, on actin dependent processes and on actin arrays. We found that NSC23766 disrupted polar secretion of adhesive, polarization of endomembranes, and tip-focused growth in the rhizoid. Similarly, NSC23766 altered actin and Arp2 localization in the growing rhizoid. In contrast, NSC23766 had no effect on selection of the growth site or on cytokinesis. These data suggest that Rac1 participates in nucleation of specific actin arrays in the developing zygote.

Keywords Actin · Actin-related protein · NSC23766 · Rac · Rho GTPase · *Silvetia*

Abbreviations
AF   After fertilization
NSC   NSC23766 (Rac1 inhibitor)

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Introduction

The establishment and expression of cellular polarity is critical for the morphogenesis and function of eukaryotic cells. In plant and other cells bound by a cell wall, cell shape is accomplished by regulating the site(s) of new membrane and wall insertion. For example, puzzle-shaped epidermal pavement cells or elongated pollen tubes achieve their unique morphologies through properly directed growth in lobes or tips, respectively. Zygotes of the fucoid (brown) algae are excellent experimental organisms for investigating the processes required for determining a growth site (Kropf 1997; Quatrano and Shaw 1997; Brownlee et al. 2001). These marine organisms grow attached to rocks in the intertidal zone along both coasts of the United States, as well as in Asia and Europe. Polarity establishment occurs in young, free-living zygotes, and generates a developmental axis, along which growth occurs several hours later. This growth axis is initially established at fertilization such that the sperm entry point defines the rhizoid pole, the future growth site (Hable and Kropf 2000). A few hours later, young zygotes firmly attach to the rocky substratum, via secretion of a polyphenolic adhesive (Vreeland et al. 1998), and monitor their local environment for spatial cues (e.g., gradients of light, temperature, ions Jaffe 1968). In response to these cues, the default sperm axis is abandoned and a new axis is established. In nature, unilateral light is likely the most important signal and the new rhizoid pole assembles on the shaded hemisphere (photopolarization). The rhizoid elongates by tip growth, and this growth axis orients the spindle which in turn specifies division transversely, generating daughter cells of different shapes as well as developmental fates. The polarly growing rhizoid cell will give rise to a holdfast, which anchors the alga to the rocky

substratum. The more symmetric thallus cell will give rise to the stipe and fronds.

In the heterokont (stramenopile) lineage, which includes the fucoid algae, actin is essential for polarity establishment and expression, yet little is known regarding its regulation. Prior to the initiation of rhizoid growth (germination) in *Silvetia compressa*, beginning approximately 4 h after fertilization (AF), a cortical patch of filamentous actin assembles at the rhizoid pole and subsequently targets accumulation of extracellular adhesive (Hable and Kropf 1998; Alessa and Kropf 1999) and endomembranes (Hadley et al. 2006). If the axis becomes reoriented, actin patch assembly, adhesive secretion and endomembrane accumulation are re-established at the new rhizoid pole. The axis becomes fixed in place at approximately 10–12 h AF, and tip growth commences at the rhizoid pole. This growth is accompanied by a reorganization of the actin patch into a cone-shaped array that extends from the nucleus to the subapex of the tip (Pu et al. 2000; Hable and Kropf 2005). The actin cone is thought to deliver secretory vesicles to the tip, where they fuse with the plasma membrane. As the tip elongates, the cone also lengthens maintaining one edge in the subapex (Hable and Kropf 2005). During cytokinesis, a plate of actin forms between telophase nuclei and grows out to meet the cortex (Bouget et al. 1996; Bisgrove et al. 2003), precisely predicting the position of new membrane and cell wall deposition (Bisgrove and Kropf 2004). Inhibitor studies suggest that the reorganization of actin during the first cell cycle is dependent on nucleation of new actin arrays, and depolymerization of the previous arrays (Quatrano 1973; Hable et al. 2003). Immunofluorescence data indicate that the cortical actin patch and the actin cone are nucleated by the Arp2/3 complex, while nucleation of the actin plate occurs independent of this complex (Hable and Kropf 2005).

In plants, the Scar/WAVE complex is a likely regulator of the Arp2/3 complex. First identified in *Dictyostelium discoideum* (Bear et al. 1998), this complex has been extensively studied in animals. To induce actin polymerization, the Scar/WAVE complex is first activated by Rac1, a member of the Rho family GTPases, which behave as molecular switches regulating numerous cellular events (reviewed in Etienne-Manneville and Hall 2002; Ridley 2006; Brembu et al. 2006). Rho proteins are activated by guanine nucleotide exchange factors (GEFs) that promote dissociation of GDP, and its replacement with GTP. These proteins are "switched" to their inactive state through GTP hydrolysis, which is stimulated by GTPase activating proteins (GAPs). Activated Rac1, interacts with the PIR 121/Sra-1 subunit of the complex which additionally contains Scar/WAVE, Nap125, HSPC300 and Abi2. This interaction activates Scar/WAVE either within the complex (Innocenti et al. 2004) or through dissociation of inhibitory subunits Pir

121/Sra-1, NAP1 and Abi1 (Eden et al. 2002; Gautreau et al. 2004). Through the highly conserved VCA domain, Scar/WAVE then recruits and activates the Arp2/3 complex.

While plant genomes lack obvious homologs of Rac1, they contain a unique sub-family of Rho-type GTPases (Brembu et al. 2006). Members of this sub-family are most similar to each other, but show greater similarity to animal Racs than to other animal sub-families, and are therefore designated RAC/Rops (Rho of plants). Homologs for all components of the Scar/WAVE and Arp2/3 complexes have been identified in plants (Frank and Smith 2002; Mathur et al. 2003a; Mathur et al. 2003b; Le et al. 2003; Li et al. 2003; Brembu et al. 2004; Deeks et al. 2004; Frank et al. 2004; El-Din El-Assal et al. 2004; Basu et al. 2005). Numerous genetic and biochemical studies have implicated RAC/Rops, the Scar/WAVE complex, and the Arp2/3 complex in control of actin and cell shape in root hairs, pollen tubes, epidermal pavement cells, and trichomes (reviewed in Brembu et al. 2005; Szymanski 2005; Hussey et al. 2006; Nibau et al. 2006).

A Rac1 homolog (FdRac1) has been identified in the brown alga, *Fucus distichus*, which is in the same family as *S. compressa*. Unlike the plant RAC/Rops, FdRac1 falls into the Rac sub-family (Fowler et al. 2004) with 84% similarity to a Rac1 homolog in humans. FdRac1 is present throughout the period of polarization, and it localizes to the cortex of the rhizoid, suggesting that it may regulate tip growth, possibly by organizing the actin cone (Fowler et al. 2004).

To determine whether Rac1 regulates Arp2/3 complex-mediated nucleation of actin in *S. compressa*, we tested the effects of Rac1 perturbation on actin-dependent processes during the establishment and expression of cell polarization. NSC23766 (NSC) is a membrane permeant chemical from the National Cancer Institute that fits the GEF recognition groove of Rac1, where it specifically inhibits activation of Rac1 in vitro, and blocks Rac1-dependent lamellipodia formation in animal cell culture (Gao et al. 2004). NSC does not affect the activity of related Rho GTPases such as Cdc42 or RhoA in vitro or in vivo (Gao et al. 2004). Specificity of NSC for Rac1 is mediated by four conserved amino acids (Lys5, Val7, Thr58 and Ser71) that line the GEF recognition groove (Gao et al. 2004); these residues are present in FdRac1. Given that FdRac1 is overall so similar to mammalian Rac1 and contains all four conserved NSC-binding residues, it is likely, though not definitively proven, that this chemical behaves similarly in the fucoid algae, inhibiting Rac1 function. We show that NSC disrupts polar secretion of adhesive, polarization of endomembranes and tip growth in a dose-dependent manner. In contrast, NSC has no effect on photopolarization or cytokinesis. Moreover, NSC treatment alters the actin/Arp2 cone in the growing rhizoid tip.

Materials and methods

Algal material

Sexually mature receptacles of the monoecious species, *S. compressa* (J. Agardh) E. Serrao, T. O. Cho, S. M. Boo et Brawley (Serrao et al. 1999) were collected near Pigeon Point Lighthouse, north of Santa Cruz, CA, USA. Receptacles were shipped cold and stored in the dark at 4°C for up to 3 weeks. To induce the release of gametes, receptacles were potentiated by placing them in uniform light (100 µmol photons m^{-2} s^{-1}) at 16°C in artificial sea water (ASW: 0.45 M NaCl, 10 mM KCl, 9 mM CaCl$_2$·2H$_2$O, 30 mM MgCl$_2$·6H$_2$O, 16 mM MgSO$_4$, and 40 µg/ml chloramphenicol, buffered to pH 8.2 with 10 mM Tris base) until they were observed to accumulate small gas bubbles on their surfaces (4 h to overnight). Potentiated receptacles were rinsed with ASW and transferred to the dark for 1 h, during which time gamete release occurred. The time of fertilization was considered to be 30 min after transfer to the dark. Zygotes were obtained by filtration through nylon mesh, rinsed two to three times with ASW, plated on coverslips that had been attached to the bottoms of petri dishes with clay, and cultured in ASW at 16°C either in unilateral light (100 µmol photons m^{-2} s^{-1}) or in the dark.

NSC23766 (Calbiochem, EMD Chemicals, San Diego, CA, USA) was dissolved in dH$_2$O at a stock concentration of 100 mM, and used at final concentration of 50–200 µM. The volume of stock added to reach the final concentrations was small (<0.2%), and this percentage of dH$_2$O does not effect development. For most experiments, zygotes were observed by brightfield microscopy on an Olympus IX51 microscope, and images were captured with an Olympus DP70 digital camera.

Adhesive labeling

Zygotes were plated on poly-L-lysine-coated coverslips (50 µg/ml; Sigma-Aldrich, St Louis, MO, USA; Hable and Kropf 1998) that had been attached to the bottoms of petri dishes with clay. After treatments, adhesive was labeled with microspheres (Polysciences, Warrington, PA, USA) which spontaneously stick to its surface, as described previously (Hable and Kropf 1998). Adhesive morphology was observed by brightfield microscopy; adhesive was identified as the clear area between the cell wall and the microsphere outline.

Endomembrane labeling

Treated zygotes were labeled with 5 µM FM4-64 (Molecular Probes, Invitrogen, Carlsbad, CA, USA; stock = 20 mM in DMSO) for 30 min and observed by conventional epi-fluorescence microscopy, using a 577.5–632.5 nm band pass emission filter (Chroma Technologies, Brattleboro, VT, USA).

Photopolarization

Zygotes were cultured on coverslips in unilateral light (L1) from 0 to 7 h AF. At 5 h AF, 0–200 µM NSC was applied, and at 7 h AF, the direction of light was reversed 180° (L2); zygotes were repolarized in L2 until 12 h AF. NSC was then replaced with ASW through several rinses, and zygotes were placed in darkness to germinate. The ability of zygotes to respond to the second light cue was determined by the direction of rhizoid germination at 24 h AF. The percent axis repolarization was calculated according to the following equation: $(L2 - L1)/(L2 + L1) \times 100$, where L2 is the number of zygotes germinating on the hemisphere shaded during L2, and L1 is the number of zygotes germinating on the hemisphere shaded during L1. Photopolarization of 100% indicated that every zygote polarized according to L2, 0% indicated random polarization of the population, and −100% indicated that every zygote polarized according to L1.

Germination

Zygotes were polarized in unilateral light from 1 to 6 h AF at which time 0–200 µM NSC was added. Zygotes exhibiting any degree of rhizoid outgrowth at 24 h AF were scored as germinated. Rhizoid morphology was quantified by determining the ratio of rhizoid length (from the point of outgrowth to the tip) to rhizoid width (the widest region of the rhizoid).

Tip growth

Zygotes were grown in unilateral light for 24 h at which time they were imaged, and embryo lengths were measured. These embryos were then either left untreated or treated with 50–200 µM NSC, and cultured for another 24 h. At 48 h AF (24 h of inhibitor treatment) the same embryos were imaged and their lengths recorded. This process was repeated once more (after 48 h of inhibitor treatment), again returning to the same embryos so that growth rates for each embryo could be determined. For each treatment, average growth rates for the first and second 24 h of treatment (from 0 to 24 h of treatment, and from 24 to 48 h of treatment) were calculated.

Actin/Arp2 labeling

Zygotes were immunolabeled as described previously using a monoclonal antibody to chicken gizzard actin (C4,

Abcam Inc., Cambridge, MA, USA) and a polyclonal antibody generated against a peptide corresponding to the C-terminus of *S. compressa* Arp2 (Hable and Kropf 2005). Fluorescence images were obtained on a LSM510 (Carl Zeiss Inc., Thornwood, NY, USA) laser-scanning confocal microscope using 488- and 543-nm laser lines with 560–600 nm narrow band pass emission filter. No bleed-through between channels was observed.

Cytokinesis

Zygotes were polarized in unilateral light from fertilization until 18 h AF, at which time media was replaced with 0–200 μM NSC. At 25 h AF, membranes were labeled with FM 4-64 and imaged as described for Endomembrane Labeling. Zygotes exhibiting a partition membrane between rhizoid and thallus were scored as having completed cytokinesis.

Results

NSC delocalizes and reduces adhesive secretion

Secretion of adhesive at the rhizoid hemisphere is an early manifestation of the growth axis, and is dependent on a dynamic population of actin (Hable and Kropf 1998). To test the role of Rac1 in polar secretion of adhesive, young zygotes were treated with 0–200 μM NSC23766 (NSC), a Rac1 inhibitor, from 1 to 6 h AF in unilateral light. Adhesive was then visualized by outlining it with microspheres.

The majority of untreated zygotes exhibited adhesive that was thickest at the rhizoid hemisphere (Fig. 1a, Table 1). In contrast, less than half of zygotes of the same age that had been treated with the lowest concentration of NSC had polar adhesive. Instead, adhesive morphology for these zygotes was variable and was scored as delocalized, uniform or undetectable (Fig. 1b–e). Increasing the NSC concentration exacerbated the effect on secretion (Table 1). Together, these findings suggest that Rac1 is required for polar secretion of adhesive.

During normal development, the rhizoid–thallus axis remains labile until just prior to germination, and throughout this time it can be reoriented by environmental cues (Jaffe 1958; Kropf et al. 1999). Since axis reorientation is dependent on a dynamic population of actin (Hable et al. 2003), NSC's effects on polar adhesive secretion were potentially due to an inability to successfully reorient the axis according to a unilateral light cue. To test this possibility, zygotes were photopolarized with unilateral light from one direction (L1) and then were repolarized with unilateral light from the opposite direction (L2) either in ASW alone or in the presence of NSC. Photopolarization was scored with respect to L2 such that 100% polarization indicated that all zygotes responded to L2. NSC had no effect on repolarization as zygotes were equally able to respond to L2 regardless of treatment: 0 μM NSC (76 ± 2%), 50 μM NSC (75 ± 7%), 100 μM NSC (76 ± 6%), 200 μM NSC (77 ± 10%). Data are average repolarization values for three experiments ± SE ($n = 568$–630 zygotes for each treatment). Thus, NSC's effects on polar secretion of adhesive
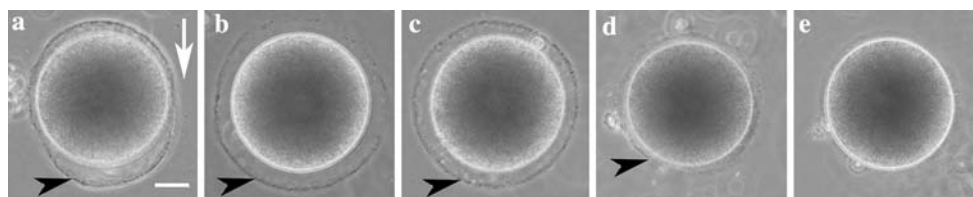

Fig. 1 Patterns of normal (**a**) and aberrant (**b–e**) adhesive secretion seen with NSC treatment. These effects are quantified in Table 1. **a** By 6 h AF adhesive is normally preferentially secreted at the rhizoid hemisphere. **b** Adhesive is modestly thicker at rhizoid hemisphere, but generally delocalized. **c** Uniform, completely delocalized secretion of adhesive. **d** Uniform and reduced secretion of adhesive. **e** No adhesive secreted. *White arrow* indicates direction of light, *black arrowheads* mark edge of adhesive. *Bar* 25 μm

Table 1 Effect of NSC on polar secretion of adhesive

	Polar	Delocalized	Uniform (thick)	Uniform (thin)	None
ASW	77 ± 1.15	7 ± 1.76	4 ± 1.15	8 ± 4.36	4 ± 1.45
50 μM NSC	43 ± 12.02	32 ± 10.8	10 ± 4.21	4 ± 3.33	12 ± 0.96
100 μM NSC	37 ± 17.34	10 ± 5.49	4 ± 1.86	25 ± 12.81	25 ± 11.79
200 μM NSC	8 ± 7.17	2 ± 2.33	1 ± 0.67	28 ± 13.48	61 ± 22.37

Zygotes were treated with control or NSC from 1 to 6 h AF, at which time adhesive was outlined with microspheres. Data represent the mean percentages ± SE for three experiments ($n = 300$–400 for each concentration). Results for each treatment were significantly different from those for the ASW control ($P = 2.05E{-}27$, $2.3E{-}35$, $5.53E{-}200$, Chi-Square test)

are not due to an inability to orient the growth axis in response to light.

NSC delocalizes endomembranes

Concurrent with polarization of adhesive secretion, endomembranes accumulate preferentially at the rhizoid hemisphere, presumably in preparation for tip growth, and actin is required to maintain this polarity (Hadley et al. 2006). To determine whether Rac1 activity is also required to maintain endomembrane polarity, 6 h zygotes were treated with 0–200 µM NSC for 2 h and endomembranes were visualized with the vital membrane dye, FM 4-64. FM4-64 intercalates into the plasma membrane where it is internalized by endocytosis and thus labels the endomembrane population. In a dose-dependent manner, NSC reduced the percent of zygotes with polar endomembranes (Fig. 2c). Eighty percent of control zygotes exhibited an accumulation of endomembranes at the rhizoid hemisphere (Fig. 2a) in contrast to 45% of zygotes treated with 200 µM NSC. Instead, 55% of these treated zygotes (and 20% of control zygotes) showed a uniform distribution of endomembranes (Fig. 2b). Lower concentrations of NSC, had reduced, but detectable, effects on endomembrane polarization. These results suggest that Rac1 plays a role in organization of membranes.
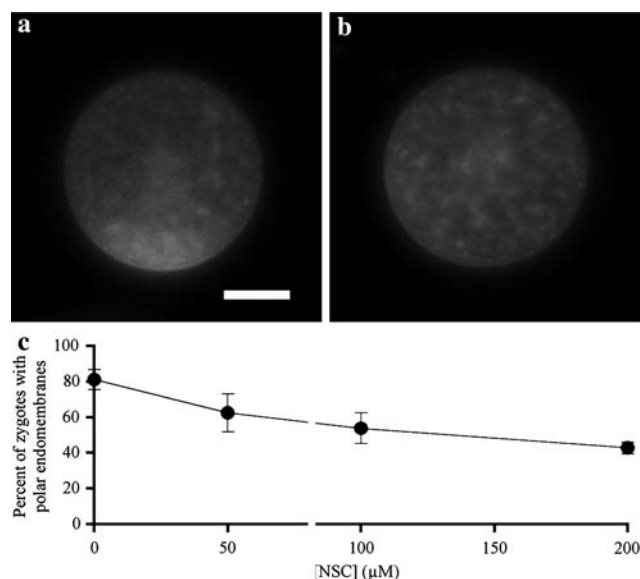

Fig. 2 Effect of NSC on endomembrane polarity. Zygotes were grown in unilateral light until 6 h AF, at which time NSC or control treatment (ASW alone) was applied. At 7.5 h AF, the fluorescent vital dye FM 4-64 was added, and zygotes were imaged at 8 h AF. **a** Control zygote at 8 h AF showing polarized endomembranes. **b** NSC-treated zygote of same age with non-polarized endomembranes. **c** Mean percentage ± SE of zygotes exhibiting polar endomembranes as a function of NSC concentration ($n = 295$–300 for each concentration, data represent three experiments). Results for each treatment were significantly different from those for the ASW control ($P = 1.87E-6$, $2.73E-20$, $1.62E-22$, Chi-Square test). *Bar* 25 µm

NSC reduces germination, tip growth and alters rhizoid morphology

The processes of rhizoid germination and subsequent tip growth are both dependent on dynamic actin (Hable et al. 2003) and are accompanied by a cone-shaped array of filamentous actin that extends between the nucleus and subapex of the tip (Pu et al. 2000; Hable and Kropf 2005). As rhizoid growth proceeds, the dynamic actin cone lengthens to keep up with the elongating tip and its edge remains in the subapex (Hable and Kropf 2005). We tested whether NSC disrupts germination and tip growth and whether it perturbs this actin array.

NSC's effect on germination was determined by treating zygotes from 6 to 24 h AF and then scoring for the presence of an emerging rhizoid. Nearly 100% of control zygotes were well-germinated by this time, while the highest concentration of NSC reduced germination to 30% (Fig. 3a, d, f). Although 50 and 100 µM NSC had little effect on the incidence of germination, rhizoid morphology was obviously altered (Fig. 3b, c). The emerging tips were more blunt than controls, exhibiting delocalized expansion, as indicated by a concentration-dependent decrease in the rhizoid length to width ratio. Average rhizoid length to width ratios ± SE of the mean were 0.83 ± 0.02, 0.78 ± 0.02, 0.63 ± 0.03, 0.42 ± 0.04 for 0, 50, 100 and 200 µM NSC, respectively ($P < 0.0001$, ANOVA). High concentrations led to lysis of some rhizoid tips (Fig. 3e).

Delocalized expansion suggests that tip growth may be compromised, so we tested the effect of NSC on growth rates of older embryos. Zygotes were grown in unilateral light for 24 h and then treated with NSC. At this developmental stage, nearly all expansion occurs in the elongating rhizoid tip, so a change in embryo length largely, if not entirely, reflects lengthening of the rhizoid. Embryo lengths were recorded for the same embryos at the start of treatment, after 24 h of treatment, and after 48 h of treatment. For each embryo, growth rates were determined for the first and second 24 h of treatment (from 0 to 24 h of treatment, and from 24 to 48 h of treatment). Control embryos grew at an average rate of 1.7–2.3 µm/h, slowing slightly in the second day. NSC inhibited tip growth in a dose-dependent manner in both the first and second 24 h periods (Fig. 4). The effect was much stronger during the second 24 h of treatment; with the highest concentration of NSC reducing the average growth rate to 0.4 µm/h, less than one-third the rate of growth in control embryos for the same time period. As with NSC's effects on germination, rhizoid expansion was also more diffuse, particularly at higher concentrations, and at the highest concentration, many tips lysed by the second day of treatment (Fig. 4c,d). Thus, it appears that the primary effect of NSC is to delocalize growth, and this then leads to shorter rhizoids.
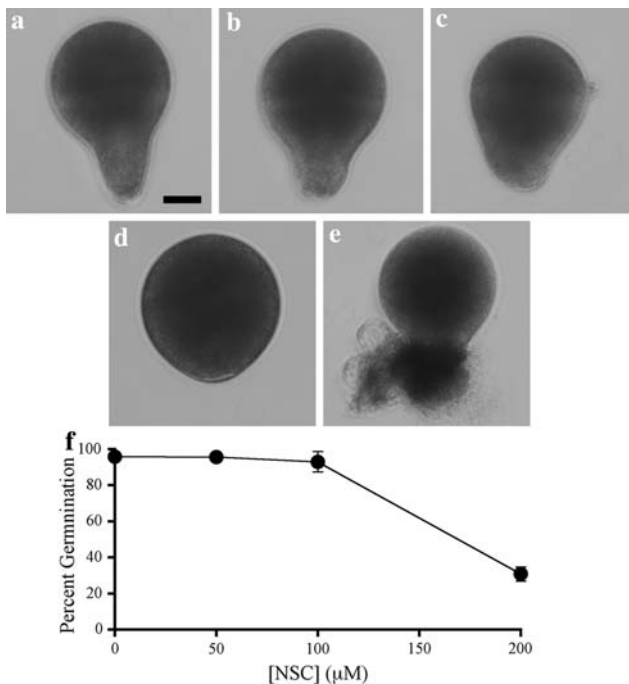
Fig. 3 Effect of NSC on germination and rhizoid morphology. Zygotes were grown in unilateral light until 6 h AF, at which time NSC or control treatment was applied. At 24 h AF, rhizoid morphology was observed and germination was assessed. **a** Untreated control with long narrow rhizoid. **b–d** Fifty, 100 and 200 μM NSC, respectively. **e** Zygote with a lysed rhizoid (200 μM NSC). **f** Mean percentage ± SE of zygotes germinating as a function of NSC concentration (*n* = 321–373 for each concentration, data represents three experiments). Results for 50 and 100 μM NSC were not significantly different from the ASW control result (*P* = 0.96 and 0.17, respectively, Chi-Square test) while results for 200 NSC were significantly different from the ASW control (*P* = 1.37E-224, Chi-Square test). *Bar* 25 μm

Fig. 4 Effect of NSC the rate of tip growth. Zygotes were grown in unilateral light for 24 h, at which time fields of embryos were imaged, their lengths recorded, and NSC or control treatment was applied. The same embryos were imaged after 24 and 48 h of treatment, and their lengths recorded. Fields of embryos after 48 h of treatment with ASW alone (**a**), 50 μM NSC (**b**), 100 μM NSC (**c**) and 200 μM NSC (**d**). **e** Mean growth rate ± SE in microns per h after 24 h of treatment (*black bars*) or 48 h of treatment (*gray bars*). Shown are results from one experiment, *n* = 17–27 embryos for each concentration. Similar trends were observed in two additional experiments. For each period of treatment, growth rates were significantly different from each other (*P* < 0.0001, ANOVA). *Bar* 50 μm

NSC disrupts actin and Arp2 localization in the rhizoid tip

In animal cells, activated Rac1 (or activated RAC/Rop in plant cells) associates with a Scar/WAVE complex of five proteins to recruit and activate the Arp2/3 complex. Once activated, the Arp2/3 complex nucleates actin filaments. To investigate Rac1's role in regulating the Arp2/3 complex and actin during tip growth in *S. compressa*, and to determine whether NSC's delocalization of tip growth is due to perturbations in the tip-localized actin array, we tested the effect of NSC on localization of actin and Arp2 in the growing rhizoid by immunofluorescence labeling. Zygotes were grown until 24 h AF at which time they were treated for 4 or 24 h and then sampled for immunofluorescence labeling. Control embryos (Fig. 5a, b) exhibited an array containing both actin and Arp2 which extended from the rhizoid nucleus to the subapex of the tip. Within the tip apex, the levels of both proteins were greatly reduced, as seen previously in germinated zygotes and embryos (Hable and Kropf 2005). This region coincides with the site of membrane insertion into the growing tip and is likely packed with vesicles (Hable and Kropf
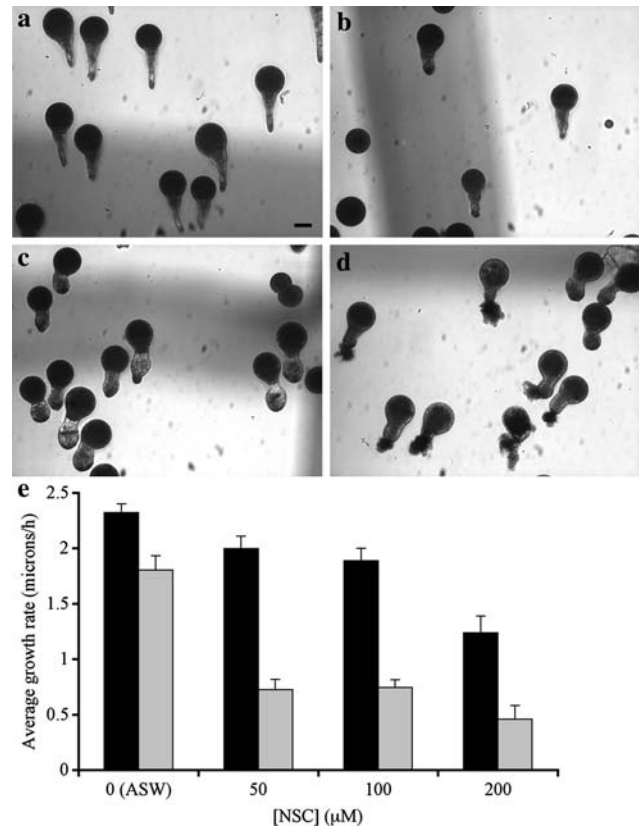
2005). In contrast, NSC-treated embryos exhibited larger, more expanded apical regions nearly devoid of actin and Arp2 (Fig. 5c–f). Instead, the actin/Arp2 array was displaced from the bulbous apex, and appeared to be unable to keep up with the growing tip. These observations suggest that Rac1 regulates tip growth through Arp2/3 complex-mediated actin nucleation, and suggest that the abnormal actin/Arp2 arrays in treated cells may no longer properly restrict vesicle fusion to the apex. The enhanced uniform cortical labeling (Fig. 5c, d) was observed variably in both control and NSC-treated embryos and is therefore not a function of the NSC treatment.

NSC does not inhibit cytokinesis

Previous experiments have demonstrated that although actin at the cytokinetic plate is dynamic (Hable et al. 2003), its nucleation does not appear to involve the Arp2/3 complex
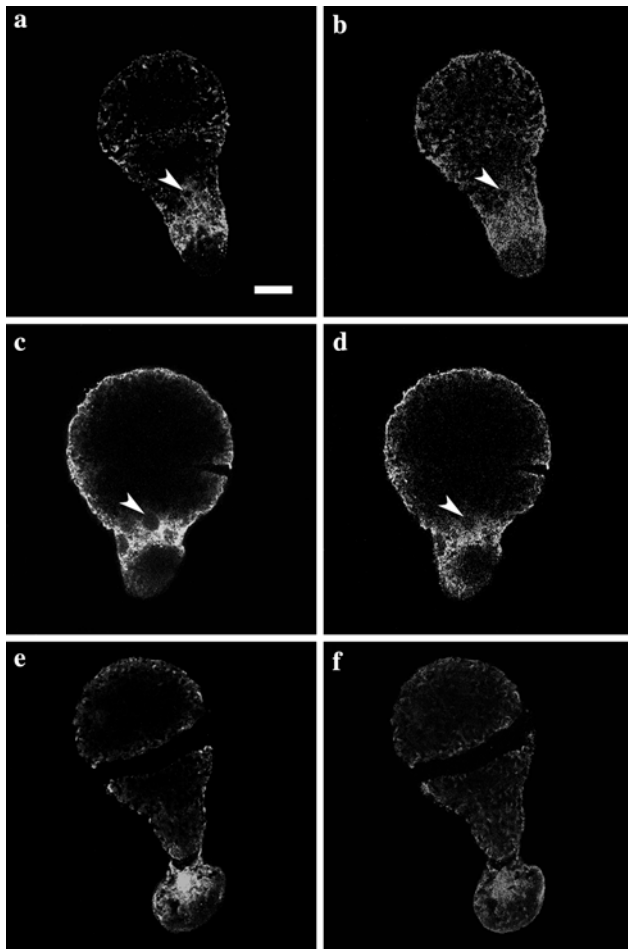
Fig. 5 Effect of NSC on actin and Arp2 localization in the rhizoid tip. Zygotes were grown in unilateral light for 24 h, at which time NSC or control treatment was applied. At 4 and 24 h after treatment, embryos were chemically fixed and labeled with actin (**a**, **c**, **e**) and Arp2 (**b**, **d**, **f**) antibodies. Embryos were imaged by laser scanning confocal microscopy. **a**, **b** Control embryo after 4 h of treatment. **c**, **d** Embryo treated with 200 μM NSC for 4 h. **e**, **f** Embryo treated with 100 μM NSC for 24 h. *Arrowhead* marks the rhizoid nucleus. *Bar* 25 μm

as Arp2 does not localize to the actin plate (Hable and Kropf 2005). Since Rac1 regulates actin through the Arp2/3 complex in plants and animals, Rac1 function may not be needed for cytokinesis in *S. compressa*. To test the possibility that Rac1 has any role in cytokinesis, zygotes were treated at 18 h AF (after germination but prior to cytokinesis) and then membranes were labeled with FM 4-64 to visualize the cytokinetic plate at 25 h AF, when normal populations of zygotes have typically completed cytokinesis. Zero to 1% of pretreated controls, sampled at 18 h AF, had divided; in contrast, by 25 h AF 98 ± 1% of control zygotes had divided and 95 ± 2% of 200 μM NSC-treated zygotes had divided. Data are averages of three experiments ± SE ($n = 300$ zygotes for each treatment). Thus, NSC not only had no effect on the incidence of cytokinesis, but it also did not appreciably delay cytokinesis.

Discussion

It is well established that actin plays a central role in eukaryotic cell polarization. It is also clear that regulation of actin arrays is controlled by a large number of proteins which constitute several possible regulatory pathways. In fucoid algae, dynamic arrays of actin are required for the establishment and expression of polarity, and immunofluorescence data suggest that the highly conserved Arp2/3 complex may nucleate many of these arrays (Hable and Kropf 2005), but which pathways regulate this complex, and how conserved are the pathways regulating this complex?

By itself, the Arp2/3 complex is an inefficient nucleator of actin polymerization (Welch 2002; Pollard and Borisy 2003). However several proteins that activate the complex have been identified (reviewed in Stradal and Scita 2006), including those of conserved WASP/WAVE family that are classified into two structural groups, WASP (Wiskott-Aldrich Syndrome protein) and Scar/WAVE (suppressor of camp receptor/WASP family verprolin homologous). Proteins of this family each have a verprolin-homology domain which binds G-actin, cofilin-homology domain and an acidic region that together bind and activate the Arp2/3 complex (Takenawa and Miki 2001). WASP itself is autoinhibited and direct interaction with the small GTPase Cdc42 or with phosphatidylinositol 4,5-bisphosphate (PIP2) disrupts this autoinhibition, resulting in activation of the Arp2/3 complex. Scar/WAVE does not have domains that bind Cdc42 or PIP3 and by itself is not autoinhibited. Instead, it is associated with a multiprotein complex, which mediates its activation by RAC/Rop (Smith and Li 2004).

In plants, homologs of the RAC/Rop-Scar/WAVE pathway have been identified, but the WASP pathway is apparently absent. Although the heterokont lineage is more closely related to plants than to animals and fungi, it diverged from green algae nearly 1,500 million years ago (Yoon et al. 2004). As a consequence, sequence similarities are relatively low, and pathways regulating actin in the heterokonts could include WASP proteins even if they have been lost from plant genomes. Genome sequencing of *Ectocarpus siliculosis*, a related brown alga, is in progress (Ectocarpus Consortium); this information will dramatically enhance our ability to determine which suites of actin-regulating proteins may be active in *S. compressa*.

To further our understanding of actin regulation during these processes, we analyzed the effects of NSC, a Rac1 inhibitor, on development and actin organization. Although it is not possible to ensure that NSC is targeting a Rac1 homolog in *S. compressa*, several lines of evidence suggest that it is likely. The related brown alga, *F. distichus* has a well-conserved Rac1 homolog which contains all four conserved amino acid residues (Lys5, Val7, Thr58, and Ser71)

that form the groove involved in NSC binding (Fowler et al. 2004). This groove normally mediates GEF binding, and the compound NSC competitively inhibits GEF association (Gao et al. 2004). Preliminary analysis of the *E. siliculosus* genome indicates at least one potential Rac1 homolog exists (unpublished observation, Ectocarpus Consortium), and using these brown algal sequences we are attempting to clone it from *S. compressa*. Moreover, in *S. compressa*, NSC disrupted several actin dependent processes and the actin/Arp2 array in the rhizoid tip. In contrast, NSC had no effect on cytokinesis which is actin-dependent but independent of the Arp2/3 complex (Hable and Kropf 2005). Finally, the concentrations used here were moderate; a previous study showed 50% inhibition of in vitro GEF binding and of nucleotide exchange at 50 μM NSC. In vivo, Rac1 activation induced by platelet-derived growth factor was inhibited dose-dependently at 50 and 100 μM (higher concentrations were not reported; Gao et al. 2004).

Our study supports a model whereby Rac1 signaling organizes actin arrays, which in turn, restrict the sites of vesicle fusion to the rhizoid pole. In young zygotes, NSC treatment depolarized endomembranes presumably leading to the observed delocalized secretion of adhesive. During germination and rhizoid growth, the inhibitor caused tips to expand non-polarly. This delocalized fusion likely occurred because aberrant actin arrays no longer adequately focused delivery of secretory vesicles toward the rhizoid pole. Further work will be required to determine how NSC treatment alters the actin and Arp2/3 complex array during polarization of endomembranes, adhesive secretion, and the initiation of germination.

The cause of tip lysis, observed after prolonged treatment at high concentrations, is unclear. Wall-loosening enzymes normally secreted in the tip may be delocalized and thus insufficient at loosening the tip. As the membrane continues to expand, increased pressure could lead to lysis. Alternatively, Rac1 may normally reinforce the tip through actin polymerization.

In germinated zygotes, the extreme apex of rhizoids is relatively devoid of cytoskeleton. This apical clear zone is thought to coincide with accumulated secretory vesicles preparing to fuse with the apical membrane. Similar to depolarization of endomembranes seen in young zygotes, NSC treatment expanded the clear zone in the tip and altered the actin/Arp2 array, resulting in delocalized tip growth. In these rhizoids, the abnormal actin/Arp2 array still delivers vesicles to the tip, but as actin polymerization is disrupted, the cone ceases to extend toward the tip, and vesicles are permitted to fuse over a greater area, creating an ever larger apical membrane.

Consistent with this model, the effects of NSC on adhesive secretion and endomembrane polarization are similar to the effects of inhibiting either actin function or secretion. Latrunculin B, cytochalasin D, and jasplakinolide, which depolymerize or stabilize actin filaments, delocalize and reduce adhesive secretion (Hable and Kropf 1998; Hable et al. 2003), and depolarize endomembrane arrays (Hadley et al. 2006). Surprisingly, even at the highest NSC concentration, at least 40% of zygotes retained polarized endomembranes suggesting that Rac1 need not be activated constantly during polarization. Inhibiting Rac1 may therefore prevent actin polymerization, but not disrupt an existing array. Brefeldin A and monensin, which block different aspects of secretion without affecting the actin cytoskeleton, delocalize, reduce or completely prevent adhesive deposition (Hable and Kropf 1998) and BFA inhibits polarization of endomembranes (Hadley et al. 2006).

As in plants and fungi, tip growth in *S. compressa* requires dynamic actin and is extremely sensitive to actin inhibitors (Hable et al. 2003). NSC disrupted both the initiation and continuation of tip growth. Although lower concentrations of NSC did not prevent germination, the morphology of the emerging rhizoid was altered, resulting in swollen tips. The elongation rate of well-germinated zygotes was inhibited in a dose-dependent manner, and again rhizoid tips swelled, indicative of delocalized, rather than tip-directed growth. These results differ from direct perturbation of the actin cytoskeleton, as both latrunculin B and jasplakinolide inhibit germination in *S. compressa* (Hable et al. 2003). However, these experiments focused on the initiation of tip growth and did not examine the effects on growing rhizoids. In *Arabidopsis thaliana* Arp2/3 complex and Scar/WAVE complex mutants have swollen trichome stalks (Frank and Smith 2002; Frank et al. 2004; Szymanski 2005), and under certain conditions, the root hairs of Arp2/3 complex mutants have a widened diameter (Mathur et al. 2003). This swollen root hair phenotype can be mimicked by low concentrations of cytochalasin D, while higher concentrations inhibit growth (Ketelaar et al. 2003). These data suggest that in some cell types, possibly including the tip growing rhizoid cell, the Rac1-Scar/WAVE-Arp2/3 pathway may serve to restrict cell expansion to the appropriate sites.

RAC/Rops comprise a multigene family of overlapping function in plants (reviewed in Nibau et al. 2006). Overexpression studies of dominant negative or constitutively active forms of RAC/Rops, as well as localization studies indicate that in pollen tubes and root hairs, activated RAC/Rops localize to the apex where they demarcate and promote cell expansion (Kost et al. 1999; Li et al. 1999; Gu et al. 2004). This well-supported model may also apply to fucoid algae since FdRac1 localizes to the rhizoid tip (Fowler et al. 2004) and our data indicate that Rac1 inhibition delocalizes growth. As mentioned previously, NSC-induced delocalized growth is also consistent with the

phenotypes seen in loss of function mutants of the Scar/ WAVE and Arp2/3 complexes.

Although Rac1 may be involved in expression of the *S. compressa* growth axis, it does not participate in axis establishment. Specifically, NSC treatment delocalized endomembranes, adhesive, and rhizoid growth, all processes guided by an established axis. Since photopolarization is dependent on dynamic actin (Hable et al. 2003) and a patch comprised of actin and Arp2 localizes to the rhizoid pole (Hable and Kropf 2005), it is somewhat surprising that NSC treatment did not prevent polarization in response to a new light cue. However, in *F. distichus*, FdRac1 is present, but not localized during polarization of young zygotes; it may not function until later (Fowler et al. 2004). It is likely that another protein, potentially WASP, activates the Arp2/3 complex during photopolarization. Whether this other protein functions alone or redundantly with Rac1 is a question yet to be addressed.

Cytokinesis requires formation of a dynamic plate of actin between daughter nuclei (Hable et al. 2003; Bisgrove et al. 2003); however, nucleation of this plate appears to be independent of the Arp2/3 complex as Arp2 is not present in this structure (Hable and Kropf 2005). Consistent with this study, we found that NSC does not disrupt cytokinesis, which proceeded in a timely fashion. Thus open questions remain concerning whether the actin plate is nucleated by a distinct mechanism, possibly including proteins in the formin or spire family.

A model for the regulation of actin arrays during cell polarization in *S. compressa* is beginning to emerge. Following photopolarization, the Arp2/3 complex nucleates a patch of actin at the rhizoid pole, and that patch guides adhesive secretion and endomembrane localization. Assuming that NSC does indeed inhibit Rac1 function in *S. compressa*, Rac1 is at least partly responsible for activating the Arp2/3 complex that nucleates the actin patch since NSC inhibited polarization of both adhesive and endomembranes. Upon initiation of rhizoid growth, the patch is remodeled to a cone that delivers vesicles to the nascent tip. Actin remodeling likely involves as yet unknown depolymerizing factors to disassemble the patch, and the new array is nucleated by Rac1-dependent activation of the Arp2/3 complex. In support of this model, NSC disrupted the actin/Arp2 cone and delocalized expansion in the tip during germination and growth. Tip localization of FdRac1 (Fowler et al. 2004) is also consistent with this result, although how it relates to actin and Arp2 localization in *S. compressa* remains to be seen. Several interesting questions remain including whether Rac1-induced activation of the Arp2/3 complex is mediated by the Scar/WAVE complex, as it is in plants and animals, and whether additional pathways activate the Arp2/3 complex, such as Cdc42 activation of WASP.

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