Activator of G-protein signaling in asymmetric cell divisions of the sea urchin embryo

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An asymmetric fourth cell division in the sea urchin embryo results in formation of daughter cells, macromeres and micromeres, with distinct sizes and fates. Several lines of functional evidence presented here, including pharmacological interference and dominant negative protein expression, indicate that heterotrimeric G protein G\(i\) and its interaction partner, activator of G-protein signaling (AGS), are necessary for this asymmetric cell division. Inhibition of G\(i\) signaling by pertussis toxin interferes with micromere formation and leads to defects in embryogenesis. AGS was isolated in a yeast two-hybrid screen with G\(\alpha_i\) as bait and was expressed in embryos localized to the cell cortex at the time of asymmetric divisions. Introduction of exogenous dominant-negative AGS protein, containing only G-protein regulatory (GPR) domains, selectively prevented the asymmetric division in normal micromere formation. These results support the growing evidence that AGS is a universal regulator of asymmetric cell divisions in embryos.

Key words: asymmetric cell division, embryo, heterotrimeric G-protein, micromere, sea urchin.

Introduction

Asymmetric cell division (ACD) is a common mechanism in the development of multicellular animals, whereby a cell produces two daughters with distinct fates. This term is applied generally, even in cases when cell divisions generate daughters of equal size, provided that distinct molecular asymmetries result, leading to cell fate diversification. ACD appears to be executed in sequential, distinct steps that include: setting up polarity in a mother cell; asymmetric distribution of determinants along the axis of polarity; orienting the mitotic spindle along the axis of polarity; executing an asymmetric division; and fixation of the distinct fates following cell division (Betschinger & Knoblich 2004).

Initial polarity in a cell is established by a conserved set of partitioning-defective (PAR) proteins in response to positional or developmental cues. The mechanism of localizing cell fate determinants is different between cell types (e.g. between C. elegans zygote and Drosophila neuroblast), but generally involves local activation of cell signaling pathways. During cell division, the mitotic spindle orients in response to this initial polarity and, in certain cases, asymmetric interaction between astral microtubules and the cortex results in unequal pulling forces acting on the forming spindle resulting in a difference in daughter cell sizes. Following cell division, unequal concentrations of determinants in daughter cells lead to different fates by such mechanisms as transcriptional and/or translational regulation (Betschinger & Knoblich 2004).

The proteins of the AGS/Pins (activator of G-protein signaling/partner of inscuteable) family are multi-domain molecular scaffolds implicated in two distinct steps of ACD execution: polarization of PAR proteins or spindle orientation and asymmetric pulling on centrosomes. AGS/Pins proteins contain G-protein regulator (GPR) motifs in the C-terminus and tetrameric peptide repeat (TPR) motifs in the N-terminus. The GPR motifs interact with G\(\alpha\) subunits of heterotrimeric complexes (e.g. G\(\alpha_i\)), while G\(i\) indicates the heterotrimetric form, complete with \(\beta\) and \(\gamma\) subunits. In addition, GPR motifs are present in other proteins, such as RGS12 or Rap1GAP. The single member of this protein family in Drosophila, Pins, was isolated in a yeast two-hybrid screen and independently as a co-immunoprecipitating partner of Inscuteable (Insc). Insc is necessary for asymmetric cell divisions of the fly neuroblasts and Pins was shown to play a role in asymmetric localization of Insc (Schaefer et al. 2000; Yu et al. 2000). Mammals appear to have two Pins...
orthologues, called LGN and AGS3. Human LGN protein (named for Leu-Gly-Asn sequences overrepresented in the TPR domains) was isolated in a yeast two-hybrid screen with Ga\(i\) (Mochizuki et al. 1996). Rat AGS3 protein was identified in a screen for cDNAs activating the yeast pheromone-response pathways in the absence of the external signal (Takesono et al. 1999), and independently by a yeast two-hybrid screen with Ga\(x\) (De Vries et al. 2000). AGS3 has a GDI (GDP dissociation inhibitor) activity towards Ga\(i\) proteins (De Vries et al. 2000); it was proposed to form a complex with an inactive GDP-bound Ga\(i\) and thus permit continuous signaling by the free \(\beta\gamma\) subunit complex. The C-terminal part of the molecule containing four GPR repeats was sufficient for inhibition of GDP dissociation from Ga\(i\) in \textit{in vitro} assays (De Vries et al. 2000). Introduction of mutations in the conserved residues of GPR motifs eliminated the interactions between AGS3 and Ga\(\alpha\) protein, and resulted in the loss of AGS3 biological activity in the yeast assay (Takesono et al. 1999).

Functional analysis of AGS/Pins in \textit{Drosophila} indicated that it is required for ACD of neuroblasts and sensory organ precursor cells (reviewed in Betschinger & Knoblich 2004). However, this specific function of AGS homologues in asymmetric divisions has so far been detected only in protostomes, while analysis of vertebrate AGS/LGN proteins in differentiated cells suggests a more general role in cell division via organization of mitotic spindles through interaction with NuMA and lethal giant larvae (Du et al. 2001; Yasumi et al. 2005). Nevertheless, the mammalian LGN protein is able to engage the cytoskeletal machinery required for asymmetric cell divisions and functionally replace Pins in \textit{Drosophila} (Yu et al. 2003b). It was recently suggested that the two vertebrate paralogues of Pins have diverged functionally, such that LGN alone is a contributor to ACD regulation. This conclusion was based on asymmetric localization of LGN in a cell cycle-dependent manner contrasted to uniform localization of AGS in dividing undifferentiated human neural progenitor cells (Fuja et al. 2004). Functionality of AGS/Pins in ACD requires the full-length protein. When the C-terminal part of AGS/Pins, containing only the GPR domains, was introduced into a cell, asymmetric distribution of the protein was lost (Yu et al. 2000, 2002), its effects on Ga\(\alpha\) function became uniform, and cell division asymmetry was disrupted (Yu et al. 2002; Cai et al. 2003).

Most of our understanding of ACD mechanisms was deduced from \textit{C. elegans} and \textit{Drosophila}, and has focused on just a few cell types: the zygote of the worm and neuroblasts and sensory organ precursors in the fly. So far, the molecular insights from these cells are not always easily translated into a greater understanding of ACD in other organisms. For example, fate determinants such as Numb, and regulators of their localization such as lethal giant larvae, play important roles in generating neuronal diversity in vertebrates, but the mechanisms of their contribution are distinct from those of the worm and fly (Betschinger & Knoblich 2004). Furthermore, an ACD execution component, LGN/AGS/Pins, seems to be regulating the organization of the mitotic spindle even in symmetric cell divisions (Du et al. 2001). Thus, a broader survey of ACD mechanisms will be valuable in identifying the general conserved features of this process. In fact, one of the well-known cases of an early ACD during embryogenesis is formation of micromeres at the 16-cell stage of a sea urchin embryo. Micromeres are a specialized cell lineage giving rise to skeletogenic mesenchyme of a sea urchin larva. This report focuses on the identification and characterization of a sea urchin AGS/Pins protein, which we found was required for normal asymmetrical division in micromere formation. The results thus suggest a broader scope for AGS in ACD events during embryogenesis.

**Materials and methods**

**Reagents**

All supplies were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted.

**Animals**

\textit{Lytechinus variegatus} was obtained from the Duke University Marine Laboratory (Beaufort, NC, USA) or collected in Tampa, FL, USA. \textit{Strongylocentrotus purpuratus} was obtained from Charles Hollahan (Santa Barbara, CA, USA). Females were shed by KCl (0.5 \(\mu\)M) injection and ovaries were then removed and minced in artificial sea water (ASW; Coral Life Scientific Grade Marine Salt; Energy Savers Unlimited, Carson, CA, USA). \textit{L. variegatus} oocytes and embryos were isolated and cultured in ASW at 22°C (Wessel et al. 2004).

**Yeast two-hybrid assay**

The Matchmaker Gal4-based Yeast Two-Hybrid System (Clontech Laboratories, Palo Alto, CA, USA) was used to screen for interacting proteins of sea urchin heterotrimeric Ga\(\alpha\) subunits. Full-length sequences of Ga\(\alpha\) subunits (Voronina & Wessel 2004b) were cloned into pGBT8 vector (Wessel et al. 2000). These bait constructs were then used to screen \textit{S. purpuratus}...
ovary cDNA library cloned into the yeast vector pACT2 (Wessel et al. 2000). The screen was performed by sequential transformation as per the manufacturer's protocol. The yeast strain PJ69-4A was transformed with the bait constructs (individually), and each carrier yeast strain was assayed for inappropriate activation of reporters (β-galactosidase expression and loss of His and Ade auxotrophy) as well as for bait protein expression (by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting). Following library transformation, the cultures were plated on Trp–/Leu–/His–/Ade– quadruple dropout media supplemented with 2 mM 3-aminotriazole. Resultant colonies were assayed by β-galactosidase colony lift assays and the inserts from the positive pACT2 plasmids were recovered by polymerase chain reaction (PCR) from the yeast genomic DNA with vector primers as per the manufacturer's protocol. These inserts were then cloned into pGEMT-Easy vector (Promega, Madison, WI, USA) and resultant plasmids were sequenced with the Gal4 activation domain primer by either the Brown University sequencing facility or Davis Sequencing (www.davissequencing.com). Additional 5’ sequences of the AGS open reading frame (ORF) were isolated by S. purpuratus ovary cDNA library (Wessel et al. 1998) screening using standard PCR-based protocols. The cDNA sequence was deposited into the GenBank (accession number DQ 358997).

RNA analysis

Reverse transcription (RT)-PCR was performed as per the manufacturer's directions using the Access RT-PCR kit (Promega), S. purpuratus AGS-specific primers (sense: GCCACATGGAGATAGCTCAAC, antisense: GTGTTCAATGGCAACTAGTTC), and S. purpuratus total RNA (1 μg/reaction), to amplify a 331 bp band from various stages of development. These primers span a 2144 bp intron in the genomic DNA (sea urchin genome project: http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). The RT reaction was performed for 45 min at 45°C; after denaturation for 4 min at 94°C, PCR amplification was performed for 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 49°C and extension for 1 min at 68°C. Ubiquitin control amplification was performed as previously described (Leguia & Wessel 2004).

Immunological procedures

To generate S. purpuratus AGS antigen, a fragment of ORF isolated in the yeast two-hybrid screen was used to produce a Glutathione S-transferase (GST)-tagged protein fusion. The GST-tagged fusion expression construct was transformed into XL1-Blue strain of E. coli and induced to express the fusion proteins with 0.5 mM IPTG for 4 h at 25°C. The cells were harvested, resuspended in the SDS-PAGE sample buffer and lysed by rapid freeze-thaw followed by sonication. Insoluble fractions were removed by centrifugation at 10,000 g for 10 min. The supernatants containing the fusion protein were loaded onto a 10% preparative SDS gel which was stained with Coomassie, except for a cut-off strip, which was blotted to nitrocellulose and probed with anti-GST antiserum. The bands corresponding to the fusion proteins were then cut out of the gel, electroeluted, extensively dialyzed against dH2O and used as immunogen to generate a rabbit polyclonal serum (as per standard procedure, Harlow & Lane 1999).

The generated polyclonal antiserum was affinity-purified on a column containing the protein immunogen. For affinity purification, 6-histidine-AGS fusion expression construct was generated (to preclude purification of anti-GST antibodies), transformed into XL1-Blue strain of E. coli and induced to express the fusion proteins as described (De Vries et al. 2000). The cells were harvested, resuspended in the denaturing lysis buffer (8 M urea, 100 mM NaCl, 20 mM Hepes pH 8.0) and lysed by rapid freeze-thaw followed by sonication. The supernatants containing fusion proteins were bound to Ni-NTA resin (ProBond, Invitrogen, Carlsbad, CA, USA) and washed with 20 mM imidazol-containing wash buffer. The bound proteins were eluted with increasing concentrations of imidazole (stepwise: 100 mM, 250 mM and 500 mM), the majority of protein eluted with 250 mM imidazole fraction. The purified protein was cross-linked to Affigel 10 as per the manufacturer's protocol (Bio-Rad, Hercules, CA, USA), and the specific antibodies were purified by sequential binding and eluting of the antibody as per the manufacturer's protocol.

Protein samples of S. purpuratus eggs and several embryonic stages were prepared by resuspending the cell pellet in SDS-PAGE sample buffer and denaturing for 5 min at 100°C in the presence of 1 mM DTT, 1 mM PMSF, 50 mM EDTA and 1 x Roche protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Samples were then subjected to SDS-PAGE followed by immunoblot analysis. For each analysis, 120 μg of each protein sample was resolved on a 4–20% PAGE gradient gel (Gradiopore, Hawthorne, NY, USA) and either stained with Coomassie blue or transferred to nitrocellulose for immunolabeling. Blots were preblocked by incubation in blotto (50 mM Tris-Cl, pH 7.5, 0.18 M NaCl, 0.05% Tween 20, 3% non-fat dry milk) for at least 15 min. Antibodies were diluted
in blotto at 7 µg/mL and applied to the nitrocellulose overnight at 4°C. The blots were then washed in blotto three times over at least 20 min. The secondary alkaline phosphatase-conjugated goat antirabbit IgG was diluted at 1:30 000 in blotto and applied to the nitrocellulose for 1 h at room temperature. Blots were washed in blotto two more times and then washed in TBST (50 mM Tris-Cl, pH 7.5, 0.18 M NaCl, 0.05% Tween 20) once, each wash lasting at least 5 min. Signal detection was carried out with BCIP/NBT development. The signal intensities were quantified using Metamorph software (Universal Imaging, Downingtown, PA, USA).

Immunofluorescence
AGS localization was performed in whole mount S. purpuratus embryos fixed in 4% formaldehyde. Samples were preblocked in 10% mouse hybridoma supernatant overnight. Antibody against AGS was diluted to 55 µg/mL in ASWT, the secondary antibody (Cy-3 conjugated affinity-purified goat antirabbit IgG; Jackson Research Laboratories, Westgrove, PA, USA) was diluted at 1:300 in ASWT. Signals were visualized and recorded by epifluorescent microscopy with a Zeiss Axioplan microscope (Zeiss, Thornwood, NY, USA) with appropriate filters.

Microinjections
L. variegatus egg and embryo injections were performed as described (Kiehart 1982; Jaffe 1999; Voronina et al. 2003). Unfertilized eggs or fertilized embryos were placed in a Kiehart chamber in ASW and injected with appropriate solutions that never exceeded 5% of the cell volume. The injection volume was kept identical between active and control reagents. An oil droplet of dimethylpolysiloxane or a 0.1 mg/mL solution of fluorescent dextran (labeled with rhodamine or Alexa Fluor 488; Molecular Probes, Eugene, OR, USA) was coinjected into cells as a marker. Both control and test reagents were injected into the same batch of cells and the cells were then incubated at 22°C. Eggs were fertilized at least 1 h postinjection.

Protein expression and purification
The 6-histidine-AGS fusion expression construct (AGS-gpr) was engineered to include the GPR domains of the yeast two-hybrid-isolated clone. The fusion protein expression was induced as described (De Vries et al. 2000). The cells were harvested, resuspended in the native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1% Tween-20, 0.1 mM EDTA, pH 8.0) and lysed by rapid freeze-thaw followed by sonication. Insoluble fractions were removed by centrifugation at 13 000 g for 30 min. Soluble fusion protein from the supernatant was purified over Ni-NTA resin as described above. For microinjection, the protein was then exchanged into injection buffer (300 mM glycine, 10 mM Hepes, pH 7.0) and concentrated with Microcon centrifugal filter devices, 10 kDa cut-off size (Millipore, Billerica, MA, USA).

Results
Gi heterotrimeric G-protein functions in early embryonic development
We have previously documented expression of the Gαᵢ subunit of the heterotrimeric G-protein complex in oocytes and eggs, as well as during embryonic development of the sea urchin (Voronina & Wessel 2004b). To test the role of Gi in the early sea urchin embryo, we injected one blastomere of the two-cell embryo with pertussis toxin, which selectively ribosylates Gαᵢ and prevents its activation in the cell. Inhibition of Gi signaling considerably interferes with cell divisions and subsequent morphogenesis (Fig. 1 and Table 1). The injected blastomeres divided slower than their non-injected sister blastomeres and did not properly form micromeres (small cells produced by the asymmetric fourth division; Fig. 1B). In blastulae, the injected portion of the embryo appeared to be disorganized and did not normally integrate into the larvae in 33% of cases, even though these cells continued to actively divide (Table 1). Recovery to a normal larval morphology 24 h following injection was observed in 67% of embryos and may be due to turnover of the modified Gαᵢ protein (Table 1). The observed effects were concentration-dependent and more pronounced in embryos treated with 1.2 µg/mL

![Fig. 1. Inhibition of Gi signaling interferes with embryonic development. A blastomere injected with 1.2 µg/mL pertussis toxin (A) does not form micromeres during the fourth division (B; a micromere formed by the non-injected part of the embryo is indicated by an arrow).](image-url)
of toxin than in those treated with 0.2 \(\mu\)g/mL of toxin. Injections of the C-terminal G\(\alpha_i\) competitor peptide, also documented to interfere with G\(\alpha_i\) activation (Voronina & Wessel 2004a), replicated the results obtained with pertussis toxin injections (Table 1). For further analysis, we focused on the micromere formation deficiency.

Identification of sea urchin AGS/Pins

To start unraveling the mechanisms of G\(\alpha_i\) signaling which are so essential for embryonic development, we sought to identify its interaction partners by a yeast two-hybrid screen. We screened a \(S.\ purpuratus\) ovary cDNA library with the three sea urchin G\(\alpha\) proteins; G\(\alpha_i\), G\(\alpha_q\) and G\(\alpha_{12}\) (Voronina & Wessel 2004b), as baits and identified three positives, among which was a sea urchin homologue of AGS. AGS was isolated as an interacting partner selectively of G\(\alpha_i\), and not other G\(\alpha\) subunits. The ORF fragment isolated in the screen was homologous to the C-termini of the LGN/AGS/Pins family of proteins containing GPR motifs: human LGN, rat AGS3 and \(Drosophila\) Pins (Mochizuki et al. 1996; Takesono et al. 1999; Yu et al. 2000; Fig. 2), and we further refer to it as AGS. Screens for N-terminal regions of AGS yielded TPR motifs as well (Fig. 2A). RT-PCR analysis of \(S.\ purpuratus\) total RNA detects AGS mRNA expression throughout sea urchin embryonic development and in adult tissues (Fig. 3). Based on observed defects in asymmetric cell divisions producing micromeres in the embryos treated with G\(\alpha_i\) inhibitors, we hypothesized that G\(\alpha_i\) together with AGS contribute to the regulation of asymmetric fourth division generating micromeres and macromeres.

Table 1. Summary of the developmental effects of pertussis toxin

<table>
<thead>
<tr>
<th>Injected reagent, concentration (no. injected embryos)</th>
<th>Normal cleavage (%)†</th>
<th>Embryos forming micromeres (%)</th>
<th>Normal blastulae formed (%)</th>
<th>Normal larvae formed (%)</th>
<th>Embryos rejecting injected tissues (%)</th>
</tr>
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<tr>
<td>Single blastomere injections</td>
<td></td>
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<tr>
<td>Pertussis toxin, 1.2 (\mu)g/mL (14)</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>Pertussis toxin, 0.2 (\mu)g/mL (5)</td>
<td>40</td>
<td>80</td>
<td>20</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Whole zygote injections</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pertussis toxin, 1 (\mu)g/mL (10)</td>
<td>60</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Pertussis toxin, 0.1 (\mu)g/mL (2)</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>G(\alpha_i) C-terminal peptide, 30 (\mu)M (6)</td>
<td>30 ‡</td>
<td>40</td>
<td>50</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

†In cases of single blastomere injection, cleavage was scored as abnormal if the injected cell was lagging behind the non-injected sister blastomere and/or if it had irregularly oriented cleavage planes. In cases of whole zygote injection, cleavage was scored as abnormal only if relative orientation of cleavage planes was disrupted (because we lacked a control sister blastomere to compare timing of cell divisions). ‡Not assessed. NA, not applicable.

Expression of AGS/Pins

To characterize expression of the AGS protein, we generated antibodies to the GPR domain portion of the protein. This antibody was then affinity-purified and it identified a single 82-kDa band by immunoblots throughout development (Fig. 3). Quantitative analysis of protein expression did not indicate statistically significant changes in the amount of protein between the stages analyzed (data not shown). We used this affinity-purified antibody to analyze the distribution of AGS protein in the developing embryo at the time of asymmetric cell divisions.

Localization of AGS in the egg was similar to that of its partner, G\(\alpha_i\) (data not shown), consistent with the interaction detected in the yeast two-hybrid screen. We focused our attention on 8-cell and 16-cell stage embryos; in which the four vegetal blastomeres each divide asymmetrically to form four large (macromeres) and four small (micromeres) daughter cells (Fig. 4). In late 8-cell stage embryos, we detected cortical distribution of the AGS in select cells of the embryo (Fig. 4A). In the embryos progressing to the 16-cell stage, loss of AGS cortical localization (and overall staining; Fig. 4C) in the newly formed micromeres was consistent with the previously observed degradation of AGS homologue LGN during anaphase to telophase transition (Du & Macara 2004). In 16-cell embryos, AGS became enriched in the micromeres, which are poised to divide asymmetrically again and form the large and small micromeres (Fig. 4E). Here, AGS is diffuse in the cytoplasm before becoming enriched at the cortex, again in agreement with the LGN pattern in interphase tissue culture cells (Du & Macara 2004).
Function of AGS in asymmetric divisions

We tested AGS function in oocyte maturation, fertilization and early embryonic cleavage by microinjecting cells with recombinantly produced GPR domains of AGS (AGSgpr, Fig. 2). When AGSgpr was injected into oocytes or eggs, no significant change in rates of oocyte maturation or fertilization were detected (data not shown). However, embryonic cell divisions were significantly affected by AGSgpr. Injection of 100–120 µg/mL final cytoplasmic concentration of AGSgpr into unfertilized eggs prior to fertilization exposed the whole embryo to the dominant-negative protein and led to disruption of cleavage in 88% of embryos on average, such as absence or delay of cytokinesis and irregular cleavage planes (17 embryos in total). These phenotypes were not observed in the embryos developing from eggs injected with the denatured reagent.

To examine the aberration more carefully, we injected AGSgpr into a single blastomere of a developing embryo. In these experiments, cleavage of the injected and non-injected halves was synchronous up to the 8-cell stage in 73% of cases on average (Fig. 5F). Following injection into one cell at the 2-cell stage, the AGSgpr construct interfered with the asymmetric cell divisions at the fourth and fifth cleavages: although the blastomeres divided, they did so without the normal asymmetry in 40% of the embryos (Fig. 5A,B) and the normal asymmetric division

Fig. 3. Activator of G-protein signaling (AGS) expression in embryogenesis. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *S. purpuratus* total RNA using AGS-specific primers detects AGS mRNA throughout sea urchin embryonic development, as well as in the adult organs. (B) One hundred and twenty µg of protein lysates were loaded per lane and probed with 7 µg/mL of anti-AGS affinity-purified antibodies; positions of molecular weight markers are noted on the left. Shown is the result of a representative experiment. T, testes; O, ovary; E, egg; 6c, 8 cell; 16c, 16 cell; B, blastula; MB, mesenchyme blastula; G, gastrula; P, pluteus; NTC, no template control amplification.
responsible for forming small micromeres instead divided symmetrically in 80% of cases. These symmetric cell divisions resulted in uniformly sized cells at the vegetal region of the injected half-embryo, in contrast to distinctly smaller micromeres and small micromeres on the control untreated side (Fig. 5D,E). Injection into one cell at the 4-cell stage disrupted both the normal asymmetrical divisions at the 16-cell stage for micromere formation and the asymmetrical division in the following cleavage that normally results in formation of small micromeres in 60% of the embryos (Fig. 5G,H). The phenotypes observed in this latter experiment were milder than those resulting from whole-egg injection. This is possibly due to cell nonautonomous effects (i.e. interactions with untreated sister blastomeres), or because the protein construct had significantly less time (∼1 h instead of 3 h) to exert its effects than when it was injected into whole eggs. Continued observation of the treated embryos indicated that the AGSgpr-injected cells continued to divide, without producing obviously asymmetric daughters (Fig. 5C,I). Furthermore, we observed defects in epithelial organization of the injected tissues at blastula (Fig. 5C,I) similar to those noted when Gi signaling was inhibited (Table 1).

**Discussion**

Two instances of cell division producing daughter cells of unequal size are well-documented in the sea urchin: formation of micromeres on the vegetal pole of the embryo at the fourth cell division and their subsequent division to form large micromeres and small micromeres. This phenomenon has been documented for over a century; however, the molecular mechanisms executing these asymmetric cell divisions remain unknown. In this report, we conclude that the asymmetric division for micromere formation requires the AGS/Gαi pathway, based on results from a combination of approaches including pharmacological inhibition of Gαi function and introduction of a dominant-negative AGS protein construct into embryos. The observed function in ACD regulation appears to be selective for regulation of cell division, because other developmental processes affected by Gi signa-
ling, such as oocyte maturation (Voronina & Wessel 2004b), were not significantly influenced by the dominant-negative AGS. In addition to disrupting the asymmetric divisions, the dominant-negative AGS protein interfered somewhat with normal symmetric cell division in the most severe cases. This is not completely unexpected: AGS/Gαi signaling has the potential to influence generic cytoskeletal machinery that executes each and every cell division, asymmetric or not. Therefore, introduction of AGSgpr protein, without its temporal and spatial regulation, leads to general disruption in cell division. Indeed, over-expression of the AGS homologue LGN in tissue culture cells disrupted general cell division as well (Du & Macara 2004). Our results indicate that the molecular mechanisms of executing asymmetric cell divisions are conserved between protostomal and deuterostomal organisms. In the characterization of deuterostomal molecular ACD regulators, AGS can now be used as a starting point to identify novel factors as well as test the function of its regulators and interacting partners known from Drosophila or C. elegans.

In the case of Drosophila asymmetric divisions, the AGS homologue Pins is used to help translate innate polarity cues into asymmetric distribution of polarity determinants. In Drosophila neuroblasts, PAR-3(Bazooka)/6 complex along with atypical protein kinase C (aPKC) and Insc enhance Pins recruitment to the apical surface of the epithelium (Yu et al. 2002; Cai et al. 2003). Formation of this PAR-3/6/Insc/Pins complex is required to maintain its apical localization and direct positioning of the mitotic spindle. In Drosophila sensory organ precursors, innate planar polarity established by Frizzled specifies anterior localization of Strabismus (Stbm), which together with Pins restricts localization of the PAR-3/6 complex to the posterior side of the cell. In a parallel pathway, Pins recruits Disks large (Dlg), which contributes to maintaining anterior/posterior polarity. In C. elegans, localization and function of GPR-domain proteins is downstream of PAR-3/6. The closest AGS/Pins orthologue of C. elegans does not function in the first-cell ACD (Betschinger & Knoblich 2004), while two additional GPR-domain containing proteins, Gpr-1 and Gpr-2, are important in generating unequal pulling forces resulting in different sizes of AB and P1 cells (Schweisguth 2000). The animal-vegetal axis of polarity is preset in the sea urchin egg and manifests itself early in embryonic development by differential stability of specific proteins along the animal-vegetal axis (Weitzel et al. 2004; Angerer et al. 2005). It is proposed that local activation of kinases such as PAR-1 might contribute to this differential stability regulation. We hypothesize that the molecular determinants of this axis mediate cortical recruitment of AGS and specify the asymmetric divisions. PAR-3 and PAR-6 proteins are present in the sea urchin genome and are good candidates to regulate the ACD generating micromeres. It remains to be seen if they are upstream or downstream of AGS/Gαi function.

Cooperation of Gαi with the AGS/Pins family of proteins in regulating asymmetric cell divisions is well-documented in the development of Drosophila and C. elegans (reviewed in Willard et al. 2004). In C. elegans, Gαi family members gpa16 and goa1, as well as Gβ1 and Gγ2 partner subunits of heterotrimeric complex, regulate spindle orientation in the first several divisions (Gotta & Ahringer 2001). In Drosophila asymmetric cell division of both neuroblasts and sensory organ precursors, heterotrimeric complex members Gαi, Gβ13F and Gγ1 are important for appropriate segregation of cell fate determinants, establishment of spindle polarity, and specification of size difference between daughter cells (Schafer et al. 2001; Cai et al. 2003). The mechanism of G-protein contribution is different in each case, such that in C. elegans, Gαi is not asymmetrically distributed and its effect is downstream of PAR. Conversely, in Drosophila, Gαi is colocalized with Pins in an asymmetric manner independent of the PAR complex in both neuroblasts and sensory organ precursors, and is required for generating wild-type distribution of not only Insc and Pins, but also PAR-3/6 and aPKC in neuroblasts (Yu et al. 2003a; Izumi et al. 2004).

In Drosophila, two redundant pathways specify asymmetric spindle positioning and size difference between daughters of dividing neuroblasts, such that predominant symmetric divisions are observed only in the case of inactivation of at least one member per pathway: PAR-3(Baz), aPKC, Insc and PAR-6 constitute one pathway, and Pins and Gαi belong to another (Cai et al. 2003). In our experiments, 60–100% of embryos exhibiting disruption of asymmetric divisions upon inhibition of solely AGS or Gαi function compare quite favorably with just 30% interference observed in the case of similar experiments in Drosophila neuroblasts (Cai et al. 2003). Potentially, the asymmetric division forming sea urchin micromeres does not rely on pathway redundancy for generating asymmetric forces pulling on the centrosomes.

Finally, the AGS/Gi pathway appears to function not only in ACD, but in stereotypically oriented cell divisions as well, such as maintaining parallel orientation of mitotic spindles to the embryo surface in dividing Drosophila epithelial cells (Izumi et al. 2004). The molecular mechanisms of AGS/Gi input into mitotic spindle orientation are still unclear, but interaction of the AGS/Gi complex with a spindle pole component
NuMA (Du et al. 2001; Du & Macara 2004) might generate pulling forces bringing the spindle poles to the cell cortex. This in turn may position the spindle through the NuMA interaction with microtubules and its associated dynein/dynactin molecular motors (Du & Macara 2004; Fant et al. 2004).

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