Untying the Gordian Knot of Cytokinesis: Role of Small G Proteins and Their Regulators

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A bility to divide is one of the basic properties of a cell. In metazoans, where cells divide in a context of germ layers, tissues, and organs, cell proliferation must be coordinated with differentiation to prevent developmental abnormalities. Genetic analyses in simple model systems (yeast, slime mold, fruit fly) have demonstrated that defects in either karyokinesis, or nuclear division (e.g., mutations that affect cell cycle checkpoints, mitotic chromosome condensation and segregation, etc.), or cytokinesis (Fig. 1), both may delay or block development.

Early studies of cytokinesis in animal cells took advantage of the ability to easily manipulate and observe large transparent eggs of marine invertebrates, such as echinoderms and ctenophores (reviewed by Rappaport, 1996). These simple but informative micromanipulation experiments have demonstrated that an actomyosin contractile ring is the driving force of cytokinesis and have led to some initial speculations about the nature of the signal inducing cytokinesis. More recently, a combination of genetic screens, genomic sequencing, and biochemical approaches have resulted in the identification of many proteins required for cytokinesis in several model organisms (Goldberg et al., 1998). Not surprisingly, many of these proteins are components of the actin cytoskeleton, actin-interacting or other structural proteins, and microtubule motor proteins. Yet, the identity of proteins required for the spatial and temporal regulation of molecular events during cytokinesis remains largely an open question. Some of the first regulatory proteins shown to be required for cytokinesis were small G proteins of the Ras and Rho (Ras homologous) families.

Small G Proteins: Janus Within

Small GTP-binding proteins (G proteins) of the Ras superfamily act at the crossroads of cell signaling pathways. They relay extracellular or intracellular signals that activate signaling networks regulating cell cycle progression, transcription, vesicle trafficking, nuclear transport, cytoskeletal dynamics, and differentiation. Like other G proteins, small G proteins cycle between inactive (GDP-bound) and active (GTP-bound) states (Fig. 2). Three classes of molecules regulate the GDP/GTP cycling. Small G proteins are activated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of bound GDP for GTP (Whitehead et al., 1997; Stam and Collard, 1999). GTPase-activating proteins (GAPs) inactivate G proteins by increasing their low intrinsic GTPase activity (Zalcman et al., 1999). In addition, inactive Rho proteins are complexed in the cytosol with guanine nucleotide dissociation inhibitors (GDIs), which keep them in an inactive soluble state by inhibiting the exchange of GDP for GTP and sequestering them from membranes (Zalcman et al., 1999).

Activation of a G protein results in a conformational change, exposing its structural domains and allowing it to interact with and activate downstream effectors. Therefore, the intracellular or subcellular concentration of the GTP-bound form determines a particular cellular response. Given the diversity of cellular and developmental roles of small G proteins one would expect that there is a complex hierarchy of molecules regulating their activity, both spatially and temporally. This may explain how the same G protein often plays multiple cellular or developmental roles. For example, developmental roles of Drosophila Rho1 range from cellularization (Crawford et al., 1998), gastrulation (Barrett et al., 1997; Häcker and Perrimon, 1998), segmentation (Mago et al., 1999), dorsal closure (Ardon et al., 1999), and cytokinesis (Prokopenko et al., 1999) to the regulation of tissue polarity (Strutt et al., 1997), and dendritic morphogenesis (Lee et al., 2000). This functional diversity may be achieved through tissue, developmental stage, or cell cycle-specific expression of regulatory molecules, such as GEFs and GAPs. An additional level of regulation of G protein signaling can be achieved through subcellular compartmentalization of the molecular machinery (upstream regulators or downstream effectors) that initiates a signaling cascade (see below).

Small G Proteins and Cytokinesis: Caught in the Act

Evidence supporting a requirement for small G proteins in cytokinesis derives from four types of experiments (Table 1). Abbreviations used in this paper: aa, amino acids; GAPs, GTPase-activating proteins; GDIs, guanine nucleotide dissociation inhibitors; GEFs, guanine nucleotide exchange factors; MLC, myosin light chain.
In Dictyostelium and Drosophila, analysis of the loss-of-function phenotype was the main experimental tool. Analyses in other organisms relied on the ability to mimic mutant phenotypes using one of three approaches: (a) overexpression of constitutively active or dominant-negative forms of small G proteins, (b) injection of antibodies or RNA interference experiments, and (c) administration (through injection, expression or addition to a culture medium) of Clostridium botulinum C3 exoenzyme. C3 is an ADP-ribosyltransferase specific for Rho (but not Rac or Cdc42) proteins. It ADP-ribosylates Rho proteins on Asn⁴¹ blocking their translocation to the plasma membrane, which effectively inhibits their biological activity (Fiorentini et al., 1998).

In all documented cases, functional or biochemical inactivation of small G proteins resulted in formation of poly-ploid cells that contained multiple (two or more) nuclei, suggesting defects in cytokinesis. However, there seem to be different requirements for Rho family members in cytokinesis in different systems, suggesting that there is a significant diversity of evolutionary roles of Rho proteins. Although to date only Rho1 has been implicated in cytokinesis in Drosophila (Prokopenko et al., 1999) and C. elegans (RHO1; Kodama, Y., A. Sugimoto, and M. Yamamoto, personal communication; Romano, A., and M. Glotzer, personal communication), both Rho and Cdc42 proteins are required for cytokinesis in X. laevis and human (Table I). In contrast to an established role of Rho and Cdc42 proteins in cytokinesis (Table I), there is only one Rac protein known to be required for cytokinesis (Dictyostelium RacE). Furthermore, the role of Ras protein in cytokinesis has been demonstrated so far only in Dictyostelium (RasG; Tuxworth et al., 1997). The involvement of Ras in cytokinesis may be restricted to one or a few phylogenetic groups, since extensive studies of Ras proteins in higher eukaryotes have not provided evidence for their role in cytokinesis.

Formation of multinucleate cells upon inactivation of small G proteins strongly suggests defects of cytokinesis. However, this has been demonstrated directly by the absence of a contractile ring or failure of a cleavage furrow in only few cases (Mabuchi et al., 1993; Drechsel et al., 1996; Gerald et al., 1998; Prokopenko et al., 1999). In addition, G proteins may regulate different steps in cytokinesis (see Figure 1).
Table I. Small G Proteins and Their Regulators Implicated in Cytokinesis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism*</th>
<th>Evidence</th>
<th>Experimental Assay</th>
<th>Observations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RasG</td>
<td>Dd</td>
<td>In vivo</td>
<td>Null mutation (gene disruption)</td>
<td>Multinucleate cells in suspension, late cytokinetic defect</td>
<td>Tuxworth et al., 1997</td>
</tr>
<tr>
<td>DGAP1</td>
<td>Dd</td>
<td>In vivo</td>
<td>Null mutation (gene disruption)</td>
<td>Increased growth rate</td>
<td>Faix and Dittrich, 1996</td>
</tr>
<tr>
<td>RacE</td>
<td>Ce</td>
<td>In vivo</td>
<td>RNAi</td>
<td>Formation of multinucleate blastomers</td>
<td></td>
</tr>
<tr>
<td>Rho1</td>
<td>Dm</td>
<td>In vivo</td>
<td>Null mutation or expression of DN Rho1</td>
<td>Failure of CR and CF, formation of multinucleate cells</td>
<td>Prokopenko et al., 1999</td>
</tr>
<tr>
<td>Pebble</td>
<td>Dm</td>
<td>In vivo</td>
<td>Null mutation or expression of DN Pebble</td>
<td>Failure of CR and CF, formation of multinucleate cells</td>
<td>Prokopenko et al., 1999</td>
</tr>
<tr>
<td>Rho</td>
<td>Cj, Sm</td>
<td>In vivo</td>
<td>Injection of C3 transferase in sand dollar eggs</td>
<td>Failure or regression of CR and CF, formation of multinucleate embryos</td>
<td>Mabuchi et al., 1993</td>
</tr>
<tr>
<td>RhoA</td>
<td>XI</td>
<td>In vivo</td>
<td>Injection of ACT Rho</td>
<td>Aberrant CF ingestion</td>
<td>Drechsel et al., 1996</td>
</tr>
<tr>
<td>Cdc42</td>
<td>XI</td>
<td>In vivo</td>
<td>Injection of ACT or DN Cdc42 in embryos</td>
<td>Aberrant CF ingestion</td>
<td>Drechsel et al., 1996</td>
</tr>
<tr>
<td>Rho D</td>
<td>Mm</td>
<td>In vivo</td>
<td>Injection of ACT Rho in Xenopus embryos</td>
<td>Failure of CF</td>
<td>Kishi et al., 1993</td>
</tr>
<tr>
<td>Rho</td>
<td>Rn, Mm,</td>
<td>In vitro</td>
<td>Injection of C3 transferase in NRK, Swiss 3T3, and HeLa cells</td>
<td>Irregular CF ingestions, ectopic cleavage sites</td>
<td>O’Connell et al., 1999</td>
</tr>
<tr>
<td>Rho A</td>
<td>Hs</td>
<td>In vitro</td>
<td>Addition of C3 transferase to culture medium of HL60 cells</td>
<td>Reduced cell proliferation, accumulation of binucleate cells</td>
<td>Aepfelbacher et al., 1995</td>
</tr>
<tr>
<td>CDC42 Hs</td>
<td>Hs</td>
<td>In vitro</td>
<td>Inducible expression of ACT CDC42Hs in HeLa-derived cells</td>
<td>Formation of giant multinucleate cells</td>
<td>Dutartre et al., 1996</td>
</tr>
<tr>
<td>ECT2</td>
<td>Hs</td>
<td>In vitro</td>
<td>Injection of anti-ECT2 antibodies or expression of DN ECT2</td>
<td>Formation of multinucleate cells</td>
<td>Tatsumoto et al., 1999</td>
</tr>
</tbody>
</table>

Rho proteins and their regulators

<table>
<thead>
<tr>
<th>Protein</th>
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<th>Experimental Assay</th>
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<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras GDI</td>
<td>Ce</td>
<td>In vivo</td>
<td>GAP assay</td>
<td>Multinucleate cells</td>
<td></td>
</tr>
<tr>
<td>GAPA</td>
<td>Dd</td>
<td>In vivo</td>
<td>Null mutation (REMI)</td>
<td>Incomplete cleavage, reversion of cytokinesis</td>
<td>Adachi et al., 1997</td>
</tr>
</tbody>
</table>

*a Species name abbreviations: Ce, Caenorhabditis elegans; Cj, Clypeaster japonicus; Dd, Dictyostelium discoideum; Dm, Drosophila melanogaster; Hs, Homo sapiens; Mm, Mus musculus; Rn, Rattus norvegicus; Sm, Scaphochelinus mirabilis; XI, Xenopus laevis. Other abbreviations: ACT, activated; CF, cleavage furrow; CR, contractile ring; DN, dominant-negative; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; GEF, guanine nucleotide exchange factor; REMI, restriction enzyme-mediated integration; RNAi, RNA interference.

*b Romano, A., T. Scheld, and M. Glotzer, personal communication.

In some cases, inactivation of a Rho protein resulted in late cytokinetic defects with incomplete or aberrant ingress (Drechsel et al., 1996; Tuxworth et al., 1997; Gerald et al., 1998; O’Connell et al., 1999) or even regression (Mabuchi et al., 1993) of the cleavage furrow. Yet, in other instances, Rho proteins were required for the initiation of cytokinesis, since the contractile ring failed to form and there were no signs of cleavage furrow ingress (Mabuchi et al., 1993; Prokopenko et al., 1999). The most compelling evidence that small G proteins are required for cytokinesis was the identification of regulators of Ras (GAPs) and Rho (GEFs and GDIs) proteins (Table I) that upon inactivation or overexpression blocked cytokinesis (Kishi et al., 1993; Faix and Dittrich, 1996; A dachi et al., 1997; Lee et al., 1997; Prokopenko et al., 1999; Tatsumoto et al., 1999; Kodama, Y., A. Sugimoto, and M. Yamamoto, personal communication; Romano, A., T. Scheld, and M. Glotzer, personal communication).

Subcellular localization studies of Rho proteins strongly support a role in cytokinesis. Rho proteins localize to the...
cytosol or plasma membrane in resting cells (A damson et al., 1992; Lang et al., 1993; Robertson et al., 1995), but translocate to the cleavage furrow and midbody during cytokinesis (Takaiishi et al., 1995; Nishimura et al., 1998). Remarkably, two Rho GEFs known to be required for cytokinesis, human ECT2 (Tatsumoto et al., 1999) and Dro sophila Pebble (Prokopenko et al., 1999), have a similar distribution during mitosis, being initially cortical or cytoplasmic and translocating to the cell equator at the onset of cytokinesis. Pebble accumulation at the cell equator parallels the assembly of the contractile ring and progression of the cleavage furrow, suggesting that it is required for the initiation of contractile ring assembly (possibly, by interacting with and activating Rho1 at the cleavage furrow). These data are consistent with the proposed GEF-dependent spatial and temporal regulation of Rho activation, leading to induction of a signal transduction pathway through a direct interaction of Rho•GTP with its downstream effectors. Transient activation of the GEF/small G protein molecular switch dependent on the targeting of proteins to a particular subcellular compartment is likely to be a very common strategy. Although the signaling pathways used by different small G proteins must be different, the basic principle of a G protein working as a switch to turn on downstream effectors remains the same and probably has been used independently multiple times during evolution to regulate a variety of aspects of cellular morphogenesis.

**Rho Signaling during Cytokinesis: The Labyrinth of Minotaur**

Among a plethora of known effectors of Rho proteins, four recently identified proteins were shown to be required for cytokinesis (Fig. 2). The formin-homology proteins, Drosophila Diaphanous (Castrillon and Wasser man, 1994; Wasserman, 1998) and its mouse homologue p140mDia1 (Watanabe et al., 1997), bind to and regulate profilin, an actin-binding protein that promotes F-actin polymerization and is required for cytokinesis (Giannanti et al., 1998; Suetusgu et al., 1999). Dictyostelium p21-activated serine/threonine kinase P A K a, a putative Cdc42/Rac effector, is thought to regulate myosin II assembly by inhibiting myosin II heavy chain kinase (Chung and Firtel, 1999). Bovine Rho-associated kinase (cleavage furrow kinase) is required for the regulation of the contractile ring contractility and for phosphorylation of intermediate filaments, leading to their disassembly and segregation into daughter cells, which, in turn, ensure efficient cell separation (Kosako et al., 1997, 1999; Y asi et al., 1998). Finally, mouse citron kinase functions at a later step by regulating actomyosin contraction in a Rho-dependent manner (M adaule et al., 1998). The role of these effectors in Rho signaling during cytokinesis is further suggested by their interaction with GTP-bound forms of Rho proteins, localization to the cleavage furrow, and colocalization with either Rho proteins or components of the contractile ring. However, a role of Rho-kinase and citron kinase in cytokinesis is suggested from experiments with dominant-negative mutants, and this conclusion awaits further proof in loss-of-function studies.

How many Rho effectors does a cell need to undergo cytokinesis? Cytokinesis is a complex event involving assembly of actin, myosin, septins, and actin-interacting proteins into a contractile ring, its dynamic contraction, and disassembly at the end of cytokinesis. Most likely, these cytoskeletal events are regulated via several signaling pathways that converge on the contractile ring, with kinases featuring prominently among Rho effectors (Fig. 2). These pathways are likely to act cooperatively, as demonstrated recently for two Rho effectors, p140mDia1 and serine/threonine kinase ROCK, in the formation of actomyosin stress fibers (Watanabe et al., 1999). Rho-activated ROCK phosphorylates and inhibits myosin light chain (MLC) phosphatase, thus promoting accumulation of phosphorylated MLC generated by MLC kinase. Phosphorylated myosin II assembles into myosin filaments and associates with actin to form stress fibers. Interestingly, the kinase domain of citron shows the highest similarity to that of ROCK (M adaule et al., 1998), though it remains to be demonstrated if citron kinase regulates myosin II polymerization during cytokinesis. Since the contractile ring is a cortical structure more complex and dynamic than stress fibers, one can expect a high degree of complexity of Rho-mediated signaling pathways regulating its function. We propose that there is an elaborate hierarchy of proteins regulating cytoskeletal dynamics at the cleavage furrow, in particular polymerization/depolymerization of molecules making up the contractile ring. Since Rho proteins, their upstream regulators, and downstream effectors all localize at the cell equator during cytokinesis, the cleavage furrow is likely to function as a workshop where protein complexes that initiate and regulate cytokinesis are assembled and disassembled.

The limited knowledge we have about signal transduction pathways that initiate and regulate cytokinesis tells us that there are at least two basic regulatory mechanisms operating during cytokinesis: (a) protein–protein interaction or binding of small molecules and (b) phosphorylation. Small G proteins undergo conformational change and become biologically active in response to GTP binding. A similar mechanism, involving protein–protein interaction, has been proposed recently for p140mDia1 (Watanabe et al., 1999). Binding of Rho•GTP to the Rho-binding domain of mDia1 is thought to disrupt the intramolecular interaction between protein termini releasing the FH1 and FH2 COOH-terminal domains required to induce actin polymerization. How common is such an activation mechanism? We know that Rho•GTP/effector interactions are necessary to initiate a signaling cascade. However, one can imagine that the signal may also be transduced via the formation of ternary protein complexes alone, without interaction-dependent conformational change as suggested for p140mDia1. Intermolecular interactions as well as conformational changes are likely to be featured in this “protein dance”. A second mechanism, likely to be universal, is regulation by phosphorylation. Kinases and phosphatases play prominent roles in downstream pathways (Rho effectors), but may also regulate the upstream components of the cytokinetic signaling machinery. ECT2 appears to be activated by phosphorylation which occurs specifically in G2/M phases and this phosphorylation is required for its exchange activity (Tatsumoto et al., 1999). Cdk1 or a Cdk1-regulated kinase may phosphorylate ECT2, since
it contains several consensus phosphorylation sites for Cdk1 (Tatsumoto et al., 1999). Interestingly, other ECT2-related RhoGEF proteins implicated in cytokinesis also contain several Cdk1 phosphorylation sites, one of which is conserved in three species (amino acids [aa] 771–774 in Drosophila Pebble, aa 671–674 in mouse Ect2, and aa 814–817 in human ECT2).

Finally, it is difficult to rationalize the unexpected cell cycle-dependent nuclear localization of three RhoGEF proteins required for cytokinesis in Drosophila and human cells (Prokopenko et al., 1999; Tatsumoto et al., 1999) or cell polarization in yeast (Cdcd24p; Toenjes et al., 1999). All three proteins localize to the nucleus in interphase cells, their levels diminish before nuclear division (or upon nuclear envelope breakdown), and proteins reappear in divided nuclei. Is it evidence for a direct link between the cytokinetic machinery and the mitotic apparatus? Or do these proteins play some role in the nucleus that is unrelated to their roles in cytokinesis? Or is it just a common mechanism to inactivate a regulatory molecule by sequestering it into the nucleus (Pines, 1999)? A nesse to these and other questions await a better understanding of the molecular pathways initiating and regulating cytokinesis.

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