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Essay

Ras-Related GTPases and the Cytoskeleton

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GTPases or GTP-binding proteins are turning out to regulate a surprisingly wide spectrum of biological processes. Peptide elongation in protein synthesis and signal transduction across plasma membranes (by EF-Tu and G proteins) are already well-known examples, but the emergence of new kinds of GTPases is creating fresh interest in unexpected places. In particular, the small GTP-binding proteins related to p21ras have been the subject of much discussion because they can regulate apparently unrelated aspects of cell biology such as growth, differentiation, and vesicle trafficking. Here I would like to focus on another recently attributed function of these small GTPases: the organization of the actin cytoskeleton.

Polymerized actin is spatially organized in all eukaryote cells into a variety of structures, such as cytoplasmic stress fibers or cables, the cortical actin network at the plasma membrane, surface protrusions (e.g., lamellipodia or microspikes), or the contractile ring formed during cell division. The roles of these actin networks are diverse. In addition to maintaining cell shape, they can be used to define cell polarity and to drive cell movement and cell division, and it should come as no surprise that actin polymerization has to be precisely controlled. The balance between monomeric and polymerized actin is known to be influenced by a large number of accessory actin-binding proteins (for review see Stossel [1989] and references therein), but recent work in yeast and in mammalian cells has revealed that the spatial organization of polymerized actin is controlled by ras-related GTP-binding proteins.

YEAST

Saccharomyces cerevisiae, despite its apparent morphological simplicity, has at least three types of polymerized actin. These are distributed asymmetrically according to the polarity of the cell, which is itself determined by the site at which the daughter cell buds from the mother during cell division—the bud site (for review see Drubin [1991]). Actin cables are found along the axis of the mother cell, actin patches are found on the surface of the bud, and a ring of actin spots forms a collar around the neck of the emerging bud. The bud site, therefore, determines the cell's polarity and the organization and distribution of the actin cytoskeleton.

Cell Polarity Genes

Genetic analysis of bud site and polarity determinants has revealed some interesting observations that are likely to have wider implications for morphogenesis and the control of actin organization in higher organisms. Five genes, *BUD* 1–5, have been identified that are required for correct positioning of the bud but not for bud assembly (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Chant *et al.*, 1991). Mutations in *BUD* 1, 2, or 5, for example, lead to random budding patterns in all cell types. Mutations in *BUD* 3 and 4 have more subtle effects; in **a** and α cells the normal axial pattern of bud formation is replaced with a bipolar pattern, whereas in **a**/α cells the mutations are silent. Another five genes that are necessary for the assembly of the bud have been identified: *CDC*24, *CDC*42, *CDC*43, and *BEM* 1 and 2 (Adams *et al.*, 1990; Johnson and Pringle, 1990; Bender and Pringle, 1991; Chant *et al.*, 1991). Mutations in these genes block budding and lead to uniform cell surface growth and a disorganized actin cytoskeleton.

How do these genes function? It is much too early to tell in any detail, but sequence analysis has been very revealing. The bud pattern gene, *BUD*1 (previously called *RSR*1) encodes a small GTP-binding protein (Bender and Pringle, 1989). It is a close relative of ras with 60% amino acid identity and appears to be the yeast homologue of mammalian rap1. BUD5 is a member of a growing family of proteins with homology to CDC25, a guanine nucleotide exchange factor for RAS, and it seems likely that BUD5 is a nucleotide exchange factor for BUD1 (Chant *et al.*, 1991; Powers *et al.*, 1991). The bud assembly gene, *CDC*42, also encodes a small GTP-binding protein. It has ~30% amino acid identity to p21ras and belongs to the rho-subfamily of ras-related proteins (Hall, 1990; Johnson and Pringle, 1990). CDC43 has been shown to encode a subunit of the geranylgeranyl transferase essential for correct posttranslational modification of CDC42 (Finegold *et al.*, 1991) whereas for *CDC*24 there is some indirect evidence that it might affect guanine nucleotide levels on CDC42 (Hart *et al.*, 1991).

This genetic analysis provides the bare bones of a model for cell polarity in yeast. BUD1 in its active GTP form determines the site of bud assembly, perhaps by recognizing a structure in the plasma membrane preserved from the previous cell cycle (e.g., the bud scar). BUD5 appears to be an activator of BUD1, and its ac-

tivity could in turn be linked to signals in late G1 of the cell cycle. More analysis will be required to determine if the other *BUD* gene products also control BUD1 activity or whether they encode structural components required for defining the new bud site. There is genetic evidence that CDC24 can interact with both BUD1 and CDC42, and perhaps this may provide a link between bud site selection and bud assembly (Chant *et al.*, 1991). In any event, CDC42 in its active GTP-bound form associates with the bud site and is able to promote the assembly of components required for bud formation. This is likely to include the chitin synthetase responsible for chitin deposition at the bud neck, as well as proteins that act as a nucleation site for the polymerization of actin into one or more of the organized structures described earlier.

Surprisingly, no GTPase *a*ctivating *p*rotein (GAP) has yet turned up in these genetic analyses. Two GAPs for RAS, IRA1, and IRA2 have been identified in yeast, and in addition to regulating RAS activity, there is now evidence to suggest that they play a more active role in the RAS/adenylate cyclase signaling complex (Tanaka *et al.*, 1990; Mitts *et al.*, 1991). Similarly there is some evidence that in mammalian cells GAP is part of the signaling complex regulated by p21ras (Hall, 1990). It seems highly likely, therefore, that GAPs will play a crucial role in the regulation of bud site selection and bud assembly. A rapGAP active on rap1, the mammalian homologue of BUD1, has been cloned from a human brain cDNA library, but it is not yet known if there is a homologous sequence in yeast (Rubinfield *et al.*, 1991). We have identified three GAP proteins in mammalian cells that are active on the CDC42 homologue, G25K/CDC42Hs (unpublished data; and Hart *et al.*, 1991): 1) rhoGAP, first identified as a GAP for p21rho and also active on p21rac; 2) bcr, the product of the breakpoint cluster region gene, first shown to be a GAP for p21rac but not for p21rho; and 3) n-chimerin, a bcr-related protein also identified as a GAP for p21rac but not p21rho (Diekmann *et al.*, 1991). Each of these proteins has a catalytic domain with ~30% amino acid identity to each other. It will be interesting to see if any bcr homologies turn up in the yeast polarity genes.

Another family of proteins identified in mammalian cells that can interact with small GTPases are the GDP-dissociation inhibitors (GDIs). In particular, a GDI specific for the rho-subfamily has been identified and cloned (Fukumoto *et al.*, 1990). This protein has the remarkable ability to extract rho-related proteins from membranes but only when they are in the GDP form, leading to the suggestion that rho-like proteins may cycle between the plasma membrane and the cytosol (Isamura *et al.*, 1990). This has important implications for the mechanism of action of the rho-like proteins including CDC42, although so far GDIs have not been reported in yeast.

Actin Polymerization Genes

The identification of loci involved in linking bud assembly to actin polymerization is less advanced. One possible candidate is an actin-binding protein, ABP1, normally localized to cortical actin but, which when overexpressed, leads to delocalized cell growth (Drubin *et al.*, 1990). A second candidate is BEM1, which has been shown to interact functionally with BUD5, and like CDC42, is essential for bud formation (Chant *et al.*, 1991). It has no sequence homology to suggest it is a regulator of CDC42 activity but instead has two SH3 domains (Chenevert *et al.*, 1992). This sequence motif was first identified in src-related tyrosine kinases, and although its function is not at all clear, it appears to be restricted to proteins that can associate with cortical actin (Koch *et al.*, 1991).

Another protein that has been linked to cell polarity and the organization of polymerized actin is the cyclase associated protein (CAP). Deletion of the C-terminal domain of CAP results in random budding and abnormal actin distribution (Gerst *et al.*, 1991). One clue to why this might happen comes from the observation that overexpression of profilin, an actin and polyphosphoinositide-binding protein, can partially overcome these effects (Vojtek *et al.*, 1991). Unfortunately as with the polarity genes, there is yet no biochemical analysis to accompany the genetics, and the chain of protein interactions from CAP to actin polymerization, perhaps via profilin, is not yet clear (Goldschmidt-Clermont and Janmey, 1991). The original interest in CAP, however, stemmed from the observation that its N-terminal domain is absolutely required for the regulation of adenylate cyclase activity by RAS (Gerst *et al.*, 1991). It has been suggested by Vojtek *et al.* that CAP may provide a link between the organization of the actin cytoskeleton and cell growth, and a search for a mammalian homologue of this domain of CAP is currently underway.

MAMMALIAN CELLS

Compared with yeast, the actin cytoskeleton of mammalian cells is much more complicated, and its precise architecture varies between cell types. Recent work, however, has provided evidence that actin organization in mammalian cells is also controlled by members of the rho-subfamily of small GTPases.

p21rho

The first suggestion that p21rho might be involved in regulating actin polymerization came from studies with a bacterial mono-ADP ribosyltransferase, C3 transferase, isolated from *Clostridium botulinum*. Introduction of this enzyme into a wide variety of cell types induces complete loss of actin stress fibers (Chardin *et al.*, 1989; Paterson *et al.*, 1990). It has subsequently been shown that C3 transferase mono-ADP ribosylates p21rho at

asparagine 41 and inactivates the protein (Sekine *et al.*, 1989; Paterson *et al.*, 1990). A complementary approach to investigate rho function taken by our laboratory was to micro-inject recombinant p21rho proteins directly into living cells. We found that this produced a dramatic increase in the number of actin stress fibers, leading us to propose that activation of p21rho stimulates the formation of stress fibers and that inactivation of p21rho (by the C3 transferase) leads to their disassembly (Paterson *et al.*, 1990). Stress fibers are dynamic structures that are influenced by a variety of signals and growth conditions. Changes in their spatial organization are often associated with cell movement, and when C3 transferase is introduced into neutrophils, the ability of these cells to respond to chemotactic agents is completely lost, presumably because of inactivation of endogenous rho protein (Stasia *et al.*, 1991).

How does rho regulate actin polymerization specifically to produce stress fibers? A more detailed analysis of rho-induced effects in cells has provided a clue. We have observed that after microinjection of rho and concomitant with stress fiber formation, the number of adhesion plaques dramatically increases (Ridley, Paterson, and Hall, unpublished data). These are clusters of integrin receptors and associated proteins, including vinculin, talin, and α-actinin, which form at points of attachment to the extracellular matrix and serve as a focal point for the formation of actin stress fibers. One obvious role of p21rho would be that of a structural component of the adhesion plaque, but this does not seem to be the case; more likely rho regulates its formation.

There are some tantalizing similarities here with other GTP-binding proteins. In particular, CDC42 is involved in regulating a similar organizational problem in yeast, namely, the assembly of bud site components at a discrete membrane site and the subsequent polymerization of actin. A more general mechanistic analogy can be drawn with elongation and initiation factors regulating protein synthesis. The elongation factor EF-Tu from *Escherichia coli*, e.g., in its GTP form binds soluble aminoacylated tRNA and transports it to the ribosome by transiently forming a ternary complex. A ribosome-induced stimulation of its GTPase activity ensures that EF-Tu.GDP rapidly dissociates, leaving behind tRNA complexed to the ribosome. In these examples the GTP-binding protein regulates the assembly of a multimolecular structure at a particular location within the cell. One possibility for rho, therefore, is that in its GTP-form it recognizes a plasma-membrane target, perhaps an integrin receptor or a cluster of receptors, and promotes the assembly of an adhesion plaque complex. This then serves as a nucleation site specifically for the formation of actin stress fibers. Evidence for this model will require a detailed biochemical analysis of rho-induced effects, but in the absence of an *in vitro* functional assay this may not be so easy.

p21CDC42Hs(G25K) and p21rac

It has been shown that yeast *CDC42* and its mammalian homologue p21CDC42Hs(G25K) are functionally interchangeable, although no biological effects of CDC42Hs in mammalian cells have yet been reported (Munemitsen *et al.*, 1990). Interestingly, a protein capable of inducing malignant transformation of NIH-3T3 fibroblasts, dbl, has homology to CDC24 and has been shown to catalyze the nucleotide exchange rate of CDC42Hs (Hart *et al.*, 1991). It is not yet clear, however, whether transformation of cells by dbl is mediated by activation of the CDC42Hs GTP-binding protein.

Our lab has obtained evidence linking a third member of this rho-subfamily of small GTPases, namely p21rac, to actin. Micro-injection of recombinant rac protein into fibroblasts stimulates the formation of actin filaments at the plasma membrane and induces membrane ruffling and macropinocytosis (Ridley, Paterson, and Hall, unpublished data). The molecular basis of membrane ruffling is not at all clear, although it is known to involve changes in the organization of cortical actin. Ruffling can be induced by a number of growth factors and by oncogenic ras protein, but we have shown that all these agents act through activation of endogenous p21rac. (Ridley, Paterson, and Hall, unpublished data).

Recently a very specialized function for p21rac has been identified in neutrophils and macrophages, and this is likely to provide a major step forward in understanding the general mechanism of action of rho-related GTPases. Stimulation of neutrophils after microbial infection induces phagocytosis of the infecting agent and activation of an NADPH oxidase to produce a potent killing agent, the superoxide radical, O_2^-. Activation of the plasma membrane-bound NADPH oxidase requires cooperation with two cytosolic proteins, p47 and p67 (for review see Morel *et al.* [1991]). Recent work with the use of an in vitro assay has revealed that p21rac, although not a stable component of this complex, is essential and is involved in promoting the assembly of the plasma-membrane complex incorporating the oxidase enzyme, p47 and p67 (Abo *et al.*, 1991; Knaus *et al.*, 1991). The availability of cloned cDNAs for both subunits of the oxidase, p47/p67 and rac, coupled with the availability of an in vitro assay, provides a unique opportunity for analyzing biochemically the function of this small GTP-binding protein.

SUMMARY

Incorporation of the available data on rac in neutrophils, CDC42 in yeast, and rho in fibroblasts suggests a general model for the function of rho-like GTPases (Figure 1). Conversion of an inactive cytoplasmic rho-related p21GDP/GDI complex to active p21.GTP occurs by inhibition of GAP and/or stimulation of exchange factors in response to cell signals. p21.GTP is then able to interact with its target at the plasma membrane. This could

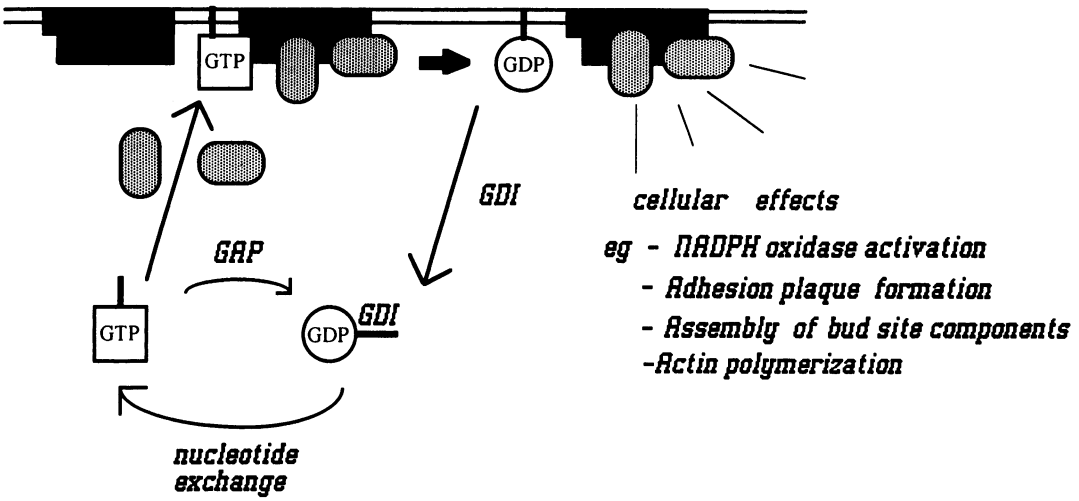

Figure 1. A model for rho-like GTPases.

result in a conformational change in the target, enabling it to bind cytosolic protein(s). Alternatively, p21.GTP could be actively involved in transporting cytosolic protein(s) to the target. A GAP protein, perhaps intrinsic to the complex, would stimulate GTP hydrolysis allowing p21.GDP to dissociate. Solubilization of p21GDP by interaction with GDI would complete a cycle.

What about the nature of the final complex? The rac-regulated NADPH oxidase complex in neutrophils is currently the best understood and most amenable to further biochemical analysis. Two plasma-membrane bound subunits encode the catalytic function necessary for producing superoxide, but the two cytosolic proteins, p47 and p67, are essential for activity. Why the complexity? Production of superoxide is tightly coordinated with phagocytosis, a membrane process driven by rearrangement of cortical actin. This is not unrelated to the membrane ruffling and macropinocytosis that we observe in fibroblasts microinjected with p21rac. It is tempting to speculate, therefore, that in neutrophils rac is involved not only in promoting the assembly of the NADPH oxidase but also in the coordinate reorganization of cortical actin leading to phagocytosis. For CDC42 controlled bud assembly in yeast, the components of the plasma-membrane complex are not so clear. By analogy with rac in neutrophils, it seems likely that CDC42 is involved in promoting the assembly of cytosolic components at the bud site on the plasma membrane. These putative cytosolic proteins have not yet been identified, but BEM1 and ABP1 are two possible candidates. The biochemical basis for the stimulation of adhesion plaques and actin stress fibers by p21rho in fibroblasts is also unclear. However, components of the adhesion plaque such as vinculin and talin are known to be cytosolic when not complexed with integrin receptors, and rho could be involved in regulating their assembly into the adhesion plaque.

Several things are still difficult to incorporate into this model. First the target for CDC42, the bud site, although not yet structurally defined requires the activity of another small GTPase, BUD1. Similarly, in activated neutrophils, the NADPH oxidase is found in a complex with rap1, the mammalian homologue of BUD1 (Bo-Koch *et al.*, 1989). It seems likely, therefore, that the target is not simply a plasma-membrane protein but may be a complex of proteins whose formation is under the control of the rap1/BUD1 GTPase.

The other black box in this model is the actin connection: activation of bud assembly by CDC42 is followed by actin polymerization, activation of NADPH oxidase in neutrophils occurs concomitantly with phagocytosis, a cortical actin-dependent process, and p21rho in fibroblasts couples the formation of adhesion plaques to actin stress fibers. One possible link between the GTPase-driven assembly of a plasma-membrane complex and actin polymerization could involve the SH3 domain. Interestingly, both p47 and p67 and yeast ABP1 and BEM1 have SH3 domains. If rho-like GTPases recognize plasma-membrane targets already associated with cortical actin, then this could promote an interaction with a subset of SH3-containing proteins. The result of this would be a GTPase-regulated aggregation of a group of proteins at a single site in the plasma membrane. It is not too difficult to imagine biological processes where such a spatial integration of different biochemical activities would be essential: coupling the assembly of bud components to the formation of actin fibers in yeast; or the activation of NADPH oxidase to phagocytosis in neutrophils; or the assembly of adhesion plaques and the formation of actin stress fibers in fibroblasts are just three examples that have emerged so far.

In conclusion, although rho-like GTPases clearly have distinct roles in different mammalian cell types and in

yeast, their underlying mechanism of action appears to be strikingly similar. Whether this will remain so when there are some biochemical data to back up these initial observations, time will tell.

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