Rho activation at a glance

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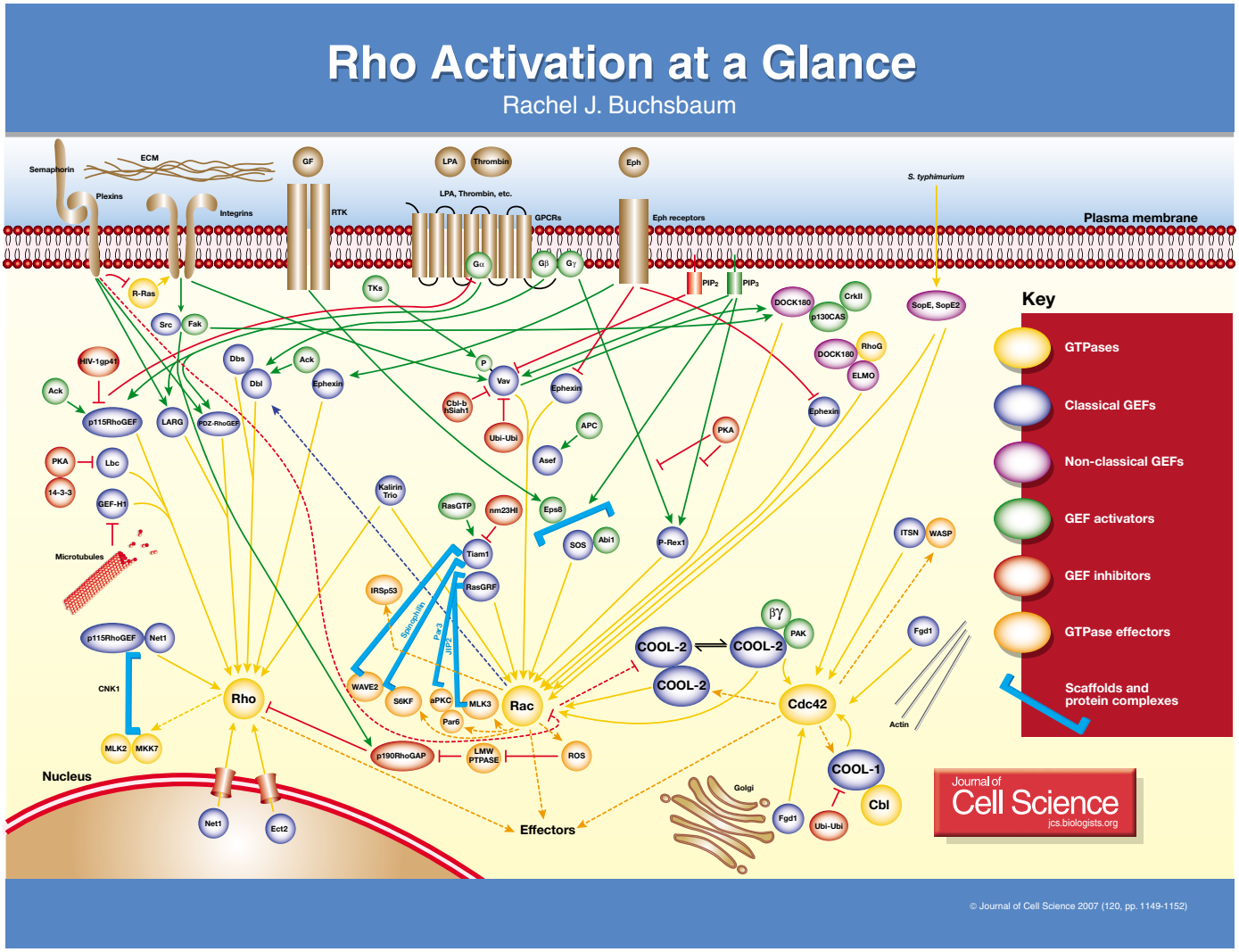
Cells receive a multitude of stimuli – chemical (such as cytokines and growth factors) and physical (such as mechanical stresses or adhesion to extracellular matrix or other cells) – that influence cell function by affecting intracellular signaling pathways. Very often these stimuli involve cell surface receptors or other molecules that function through activation of the Rho family of small GTPases. Rho GTPases control multiple cellular processes, including actin and microtubule dynamics, gene expression, the cell cycle, cell polarity and membrane transport, through their ability to bind to numerous downstream effectors, which lead to diverse parallel downstream signaling pathways (Schwartz, 2004).

Over 20 members of the Rho family have been identified in mammalian cells (Wennerberg and Der, 2005). These are represented here by the canonical proteins Rho, Rac and Cdc42 and have been the subject of numerous excellent reviews (Bishop and Hall, 2000; Ridley, 2001; Boettner and Van Aelst, 2002; Ridley et al., 2003; Burridge and Wennerberg, 2004; Jaffe and Hall, 2005). Like the classic monomeric Ras GTPase, most Rho proteins act as switches by cycling between active (GTP-bound) and inactive (GDP-bound) conformations. (Four Rho family members are GTPase deficient and bind GTP constitutively; little is known about their regulation.) There are three classes of regulatory proteins that affect the activation state of these cycling Rho molecules: guanine nucleotide exchange factors (GEFs), which promote exchange of GTP for GDP; GTPase-activating proteins (GAPs), which enhance the intrinsic GTP-hydrolysis activity, leading to GTPase inactivation; and guanine-nucleotide-dissociation inhibitors (GDIs), which bind to prenylated GDP-bound Rho proteins and allow translocation between membranes and the cytosol. Currently, the best understood regulators of Rho activation in response to upstream stimuli are the GEFs.

GEFs for Rho family proteins have been identified in bacteria, plants, yeast, worms, fruit flies and humans. Most



(See poster insert)

RhoGEF activity is mediated by catalytic DH (Dbl homology) domains, which stabilize GTP-free Rho intermediates, effectively leading to GTP loading owing to high intracellular GTP levels. DH domains contain three conserved regions and form related structures of elongated bundles of α-helices, in which amino acid variations confer specificity towards individual GTPases. Almost all DH-containing Rho family GEFs contain a PH (pleckstrin homology) domain immediately C-terminal to the DH domain. DH-associated PH domains have several regulatory roles with regard to DH domain and GEF function, including modulation of exchange activity, interactions with phospholipids and proteins, and membrane targeting.

The identification of over 60 mammalian Rho family GEFs to date has revealed a complex array of regulatory mechanisms for these proteins. The poster depicts the best-studied GEFs and major themes in their regulation. A comprehensive description is beyond the scope of this overview and I apologize to those authors whose work is not included. However, several excellent reviews have been published (Schmidt and Hall, 2002; Erickson and Cerione, 2004; Rossman et al., 2005).

A common theme in the regulation of Rho family GEFs is relief of intramolecular inhibitory interactions. This is illustrated by the constitutive activation of N-terminally truncated Dbl, Vav, Asef, Tiam1, Ect2 and Net1 mutants and C-terminally truncated p115RhoGEF and Lbc mutants. Often the associated PH domain is involved in this intramolecular inhibition. In the RhoGEF Dbl, for example, the N-terminus binds to the PH domain, blocking access of the GTPase to the DH domain. Phosphorylation by Ack1 or interaction with heterotrimeric G protein βγ subunits may relieve the inhibition. In the RacGEF Vav, an interaction between DH and PH domains that masks the Rac-binding site is induced by binding of phosphatidylinositol 4,5-bisphosphate (PIP_2) to the PH domain and relieved by binding of phosphatidylinositol 3,4,5-trisphosphate (PIP_3). Complex inhibitory intramolecular interactions have been described for the dual Ras-Rac exchange factor SOS, which possesses both a CDC25-like exchange domain for Ras as well as a DH domain to promote GTP-GDP exchange on Rac. PIP_3 binding relieves a similar DH-PH interaction that blocks GTP-GDP exchange on Rac, while the DH-PH region itself blocks an allosteric Ras-binding site on SOS that regulates Ras activity. Recently published work indicates that EGF-receptor-dependent phosphorylation of a tyrosine residue near a downstream regulatory region is required to unmask the exchange activity of the Cdc42 GEF COOL-1/βPIX (Feng et al., 2006).

GEFs have a number of other functional domains, many of which couple to upstream receptors or other signaling molecules. It is thus not surprising that protein-protein interactions also regulate GEF activity. Activated $Gα_{13}$, released from its βγ subunits by LPA- or thrombin-mediated stimulation of G-protein-coupled receptors (GPCRs), can bind to and stimulate several RhoGEFs, including Dbl, p115RhoGEF, PDZ-RhoGEF and LARG. The Gβγ units bind and activate Dbl, as mentioned above. The hematopoietic RacGEF P-Rex1 is activated by binding to both PIP_3 and specific Gβγ subunits. In the RacGEF Asef, interaction between the N-terminal ABR region and the armadillo repeat domain of the adenomatous polyposis coli protein (APC) relieves autoinhibition and promotes Rac exchange activity. Some GEFs, such as Dbl, Dbs, and RasGRF1 and RasGRF2, form homo- or hetero-oligomers through DH domain interactions that are required for full function of the protein, whereas dimerization of others (PDZ-RhoGEF, LARG, p115RhoGEF) is inhibitory. In the case of the multidomain GEFs Kalirin and Trio that contain separate DH domains for Rac and Rho, alternative splicing leads to multiple isoforms that have different functional activities during development.

GEF activity can also be downregulated by interaction with inhibitory proteins – for example, the inhibition of Vav by binding of the C-terminus to Cbl-b or hSiah1, the inhibition of p115RhoGEF by binding of the C-terminus to the HIV-1gp41 protein, and the inhibition of Tiam1 by binding of the N-terminus to the tumor suppressor nm23H1. Association of microtubules with the RhoGEF GEF-H1 inhibits its exchange activity toward Rho. Oligomerization of the A-kinase-anchoring protein (AKAP) Lbc through C-terminal leucine zipper sequences is required for inhibition of its exchange activity by PKA and 14-3-3. In addition, Rho family GEFs may also be downregulated by being targeted for degradation. Examples include SOCS1-stimulated Vav polyubiquitylation, Ras-stimulated polyubiquitylation of the dual Ras-RacGEF RasGRF2, the ubiquitylation of murine SOS2, and the Cbl-directed ubiquitylation of COOL-1/βPIX.

Multiple levels of regulation have been identified in the case of some Rho family GEFs. Interaction of the PH domain of RacGEF Vav with PIP_3, for example, allows tyrosine phosphorylation of residues interacting with the GTPase-binding pocket by Src/Syk tyrosine kinases (TKs) in response to T cell receptor signaling, further opening up access to the Rac-binding site. The activation of P-Rex1 by PIP_3 and Gβγ subunits is blunted by PKA-mediated phosphorylation, and the exchange activity of the RacGEF Tiam1 is modulated by both phosphoinositide binding and by phosphorylation on threonine and tyrosine.

Change in intracellular location and localized activation of Rho GTPases is another mechanism for regulation of Rho family GEFs. Sometimes the PH domain mediates translocation – for example, the localization of Dbl and Lbc to actin stress fibers. Some GEFs, such as Tiam1 and Fgd1 (a Cdc42GEF), contain a second PH domain. In the case of Tiam1, the second PH-domain is required for membrane translocation; in Fgd1, a proline-rich N-terminal region, rather than the PH domains, localizes the protein to subcortical actin and Golgi structures. Other Rho family GEFs are recruited to membranes by adaptor proteins, direct binding, or other protein interactions. Adaptor proteins such as Grb2 and SLP-76 are required for localization of Vav to activated B- and T-cell receptors. The DH-PH domains of ephexin directly interact with the transmembrane receptor tyrosine kinase EphA4. Binding of $Gα_{13}$ after LPA or thrombin stimulation induces redistribution of p115RhoGEF from the cytosol to the plasma membrane. And upon nuclear envelope breakdown prior to mitosis, the RhoGEFs Ect2 and Net1

are released from the nucleus, where they are otherwise sequestered, to activate the Rho-mediated contraction of the actomyosin ring that drives cytokinesis.

GTPases themselves often regulate Rho family GEF activity. For example, several RhoGEFs activated by Gα-GTP, including p115RhoGEF, LARG and PDZ-RhoGEF, also contain an RGS domain. This domain functions as a GAP for Gα subunits, thus allowing the GEFs to fine-tune signals coming from GPCRs. The RacGEF Tiam1 is activated by Ras-GTP through its Ras-binding domain. Similarly, binding of Rac-GTP to the PH domain of the RhoGEF Dbs promotes Rho activation. During the process of actin remodeling and cell spreading, Rac activation generates reactive oxygen species that inhibit the low-molecular-weight protein tyrosine phosphatase (LMW-PTPase), leading to increased tyrosine phosphorylation and activation of p190RhoGAP and subsequent downregulation of Rho activity. In a complex process probably involving both allosteric and direct effects, Cdc42-GTP activates and Rac-GTP inhibits the RacGEF activity of dimeric COOL-2/αPIX (Baird et al., 2005).

Another emerging theme in Rho family GEF function is a role specifying signaling downstream of Rho GTPases, often through participation in multimolecular complexes. This can be through direct binding of the GEF to the immediate GTPase effector protein downstream – for example, binding of COOL-2/αPIX to Pak and binding of intersectin (ITSN) to Wiskott-Aldrich syndrome protein (WASP). Alternatively this can be through binding of the GEF to scaffold proteins that also form a complex with GTPase effectors – for example, binding of Tiam1 and RasGRF1 to the p38 scaffold JIP2, and binding of Tiam1 to the F-actin–protein-phosphatase-1–S6kinase scaffold spinophilin and the Par3 component of the Par polarity complex (Mertens et al., 2006). A combination of both mechanisms is displayed by Tiam1, which can also interact with IRSp53, a small adaptor molecule that binds activated Rac as well as the Arp2/3–G-actin scaffold WAVE2. Our understanding of the complex contributions of scaffold proteins to GEF-directed GTPase signaling is just beginning. For example, the human ortholog of Connector Enhancer of Ksr1 (hCNK1), a multi-domain adaptor protein first implicated in Ras signaling pathways, may also function as a scaffold protein for Rho-dependent Jnk activation through binding RhoGEFs Net1 and p115RhoGEF, activated Rho, MLK2 and MKK7 (Jaffe et al., 2005).

In some cases where GEFs activate more than one GTPase, formation of a complex can also direct GEFs towards specific GTPases. The dual Ras-Rac-GEF SOS, for example, contains a proline-rich C-terminus that can interact with the SH3 domains of either Grb2 or Abi1/E3b1. Interaction of SOS with Grb2 enables recruitment of the complex to tyrosine kinase receptors and activation of Ras. By contrast, interaction of SOS with Abi1 leads to formation of a complex with Eps8 and activation of Rac. Similarly, the GTPase activation profile of ephexin is modulated depending on the activation state of the EphA4 receptor that it binds. When unstimulated, ephexin can activate Cdc42 and possibly Rac in addition to Rho, but EphA4 clustering leads to enhanced Rho activation and suppresses Cdc42/Rac activation. The GEF specificity of COOL-2/αPIX depends on its monomer-dimer equilibrium. As noted above, the dimer promotes Rac activation. However, interaction with Gβγ-PAK complexes promotes dissociation to monomers, which can activate either Rac or Cdc42. Finally, although Ras-GRF1 and Ras-GRF2 can each potentially activate both Ras and Rac GTPases, RasGRF1 preferentially activates Rac to promote LTD (long-term depression) and Ras-GRF2 preferentially activates Ras to promote LTP (long-term potentiation) in the hippocampus, at least in part because they are associated with different subsets of NMDA glutamate receptors in the brain (Li et al., 2006). The fact that GEFs both turn on GTPase signaling and specifically direct the downstream effects of that signaling may explain why higher organisms have evolved so many more GEFs than GTPases.

There are proteins that lack the classical tandem DH-PH motif but exhibit nucleotide exchange activity toward Rho family members. SWAP-70, part of the B cell DNA recombination complex, and related family members, possess PH domains N-terminal to DH-like domains and function upstream of Rho GTPases. In humans the CZH/DOCK180 superfamily members lack DH-PH domains altogether but possess DOCK homology domains required for nucleotide exchange through an as-yet-unidentified mechanism. Canonical members act as RacGEFs, others are Cdc42GEFs, and some remain to be characterized. Bacteria, which lack their own Rho GTPases, have multiple mechanisms for modulating the activity of mammalian Rho family proteins. The bacteria *Salmonella typhimurium* injects the non-DH-containing SopE (Rac and Cdc42) and SopE2 (Rac) proteins into mammalian cells, where they function as GEFs in order to facilitate bacterial internalization. And in plants, which contain numerous Rop (Rho of plants) proteins, a new family of Rho GEFs has recently been identified. These contain a novel PRONE (plant-specific Rop nucleotide exchanger) domain (Berken et al., 2005).

GEF activation of Rho GTPases, like all signaling pathways, does not take place in an isolated linear fashion but occurs through the serial formation of macromolecular complexes at precise points in time and space that are currently incompletely understood. Further insight into how Rho proteins are activated by upstream signals will require more knowledge of how the effects of GEFs are coordinated with those of GAPs and GDIs, along with other signaling molecules. An example of this is integrin signaling, itself a complex system involving multiple ligands and multiple receptors. There are many examples of integrin activation leading to signaling through Rho proteins, but the details of the regulatory proteins involved are often unclear. Integrin ligation is known to trigger signaling through activation of Vav and formation of DOCK180 complexes containing either p130CAS and CrkII or the Rac-like RhoG and the DOCK180-binding protein Elmo (Hsia et al., 2003; Katoh and Negishi, 2003; Gakidis et al., 2004). However, integrin signaling also leads to Rho inactivation through Src-mediated activation of RhoGAP and Rac activation through release of RhoGDI (Dib et al., 2001; Del Pozo et al., 2002).

Furthermore, pathways involving multiple GTPases are often coordinately

triggered during complex biological processes. An example of this is seen during axon guidance in the developing nervous system. Binding of the transmembrane glycoprotein semaphorin 4D to its transmembrane receptor plexin-B1 leads to Rho activation through stimulation of the RhoGEFs LARG and PRG, sequestration of active Rac by a GTPase-binding domain on plexin B1, and inactivation of the Ras family member R-Ras through a plexin GAP domain, which leads to integrin detachment, repulsion of the axonal growth cone, and turning of the developing axon (Kruger et al., 2005). Understanding the coordinated regulation of RhoGEF activity with that of GAPs, GDIs and other Ras family proteins will constitute the next frontier in the study of Rho GTPases.

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