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Structural Elements, Mechanism, and Evolutionary Convergence of Rho Protein−Guanine Nucleotide Exchange Factor Complexes

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ABSTRACT: Rho GTPases act as key regulators of cellular biochemistry by determining the timing, direction, and amplitude of signal transduction in a number of important pathways. The rate of activation of a GTPase-controlled reaction is limited by the rate of GTP binding to the Rho protein, and this, in turn, depends on the rate that GDP dissociates from the GTPase. The latter is controlled by the action of guanine nucleotide exchange factors (GEFs) that catalyze GDP−GTP exchange by increasing the rate of GDP dissociation. Here, the recently reported structural information for Rho GTPase−GEF complexes and the molecular basis for the specificity of their interactions are discussed. Underscoring the importance of regulating the Rho GTPase activation pathway, genetically unrelated proteins have evolved which complement or mimic the Dbl homology−Pleckstrin homology (DH−PH) domain-containing family of proteins in their ability to catalyze GDP−GTP exchange. In particular, the structure of the mammalian Cdc42 protein bound to the SopE protein from *Salmonella typhimurium* illustrates how two unrelated protein folds are able to carry out guanine nucleotide exchange by a remarkably similar mechanism. It will be interesting to see if this conservation of mechanism extends to a newly recognized class of GEFs related to the DOCK180 family.

The emergence of Ras-like GTPases as key players in a multitude of cellular processes has ushered in a great deal of research focused on their regulation in the cell. Members of the Rho subfamily, including Rac, Rho, and Cdc42, are involved in a number of complex signal transduction pathways that direct the remodeling of the actin cytoskeleton (*1*), influence intracellular transport (*2*, *3*), and regulate gene transcription (*4*, *5*). Some or all of these pathways may contribute to the transformed phenotype observed for cells that have been shown to highly overexpress a Rho GTPase or fail to regulate its nucleotide-bound state (*6*−*11*). This is a consequence of the cellular role of GTPases (or G-proteins) as molecular switches which cycle between GTP- and GDP-bound states and undergo corresponding changes such that they are active and able to bind downstream targets in their GTP-bound state and are inactive or incapable of signal propagation in their GDP-bound state. The rate and extent of G-protein activation via this GTP-binding/GTP hydrolytic cycle are determined by a variety of factors including intracellular localization (*12*) and extracellular stimuli (*13*) and are orchestrated at the molecular level by proteins that directly affect the rate of exchange of GDP for GTP (*14*). Overall, factors affecting the GTP-binding/GTP hydrolytic cycle of Rho GTPases include three primary regulatory proteins: the Rho guanine nucleotide dissociation inhibitors (GDIs;[1] *15*, *16*), Rho GTP hydrolysis-activating proteins (GAPs; *17*, *18*), and a growing family of guanine nucleotide exchange factors (GEFs; *14*, *19*) which bind to the GTPase

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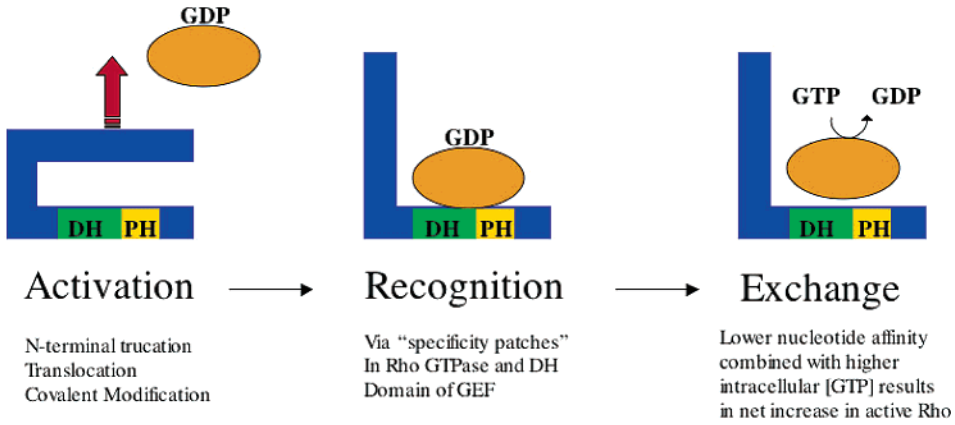

FIGURE 1: Model of Dbl family exchange proteins depicting allosteric relief of autoinhibition of nucleotide exchange activity allowing for the activation of Rho GTPases.

and, by catalyzing the exchange of GDP for GTP, promote its activation (Figure 1). The GDP−GTP exchange reaction is thought to be the rate-limiting step in the GTP-binding/GTP hydrolytic cycle of Rho GTPases, thus making it important to understand how GEFs undergo activation and how they selectively catalyze guanine nucleotide exchange on Rho proteins.

In this review, we highlight some of the progress made in understanding the molecular details of the mechanism for guanine nucleotide exchange and examine the basis of the specificity of Dbl homology (DH) domain-containing GEFs. Recent discoveries regarding genetically unrelated proteins which also possess GEF activity toward Rho-like GTPases are also discussed. The mechanistic and structural convergence of evolutionary distinct domains for Rho GEFs points to the critical importance of regulating the degree of Rho protein activation in cellular homeostasis.

Crystallographic Determination of Rho GEF Structure and the Basis for GEF−GTPase Catalytic Specificity. The GEFs for Rho GTPases take their name from the first protein demonstrating GDP−GTP exchange activity for this subfamily of G-proteins. This oncoprotein, named Dbl for *d*iffuse *B*-cell *l*ymphoma (*20*−*22*), is a prototype for a family of proteins that in most cases possess a Dbl homology (DH) domain located immediately N-terminal to a Pleckstrin homology (PH) domain (but see ref *53*). From the earliest structural studies of DH domains, it became clear that conserved regions identified by primary sequence alignment (referred to as conserved regions 1, 2, and 3) map to three conserved helices that are cylindrically packed together to form the core of the DH domain (*23*−*25*). Together, these roughly parallel helices provide a structural fingerprint for the DH domain architecture, although many of the residues necessary for GTPase binding and nucleotide exchange specificity lie outside of this region (*26*; see below). Despite the fact that, thus far, all members of the Dbl family of Rho GEFs have revealed a similarly folded DH domain structure, the precise molecular contacts responsible for the observed GEF activity remained uncertain and not obvious from primary sequence alignments alone (*23*). In the past, this limited degree of sequence conservation and the multitude of cross-reacting GEF−GTPase pairs observed in vitro and in vivo contributed to a complicated picture of GEF-dependent activation of Rho GTPases.

A wealth of structural data has recently provided the molecular details of the mechanisms used by Rho GEFs to stimulate guanine nucleotide exchange and has shed light on the differences underlying the structure−activity relationships for various pairs of Rho GTPases and GEFs. Following the initial reports describing the three-dimensional structures for the isolated DH domains of Cool/Pix (*25*) and Trio (*24*), as well as the DH−PH domains of mSOS (*23*), an X-ray crystal structure for the complex of the Rac1 GTPase and the limit DH−PH domains of the Rac-specific GEF, Tiam-1, was solved (*27*). For the first time, the sequences of Rac, Rho, and Cdc42, along with the large amount of GEF sequence information, could be viewed in the context of this crystal structure and the observed contacts of each protein in the complex. Subsequent studies suggested that the main interactions imparting specificity for the binding of Rac to the Tiam-1 DH domain involved primarily nonconserved residues in helices $\alpha_{7a,b}$[2] and the short loop just N-terminal to these residues (*28*). Evidence for the importance of this region in GEF specificity was provided by mutating Rac at residue 56 where a Trp to Phe substitution, which represents the equivalent amino acid in Cdc42, allowed Intersectin, a Cdc42-specific exchange protein, to activate Rac. Conversely, by changing the tryptophan at position 56, a network of interactions necessary for Tiam-1 recognition of Rac was lost, resulting in the complete elimination of the Rac GEF activity of Tiam-1 (*28*, *54*).

Subsequent crystal structures of Dbl family members bound to their Rho protein targets demonstrated how the DH domain recognizes, in a lock-and-key fashion, the relatively rigid structure of $\beta_2-\beta_3$ in Rho GTPases (*26*, *29*). This "specificity patch" contains all but one of the main residues in Cdc42, Rac, or Rho that make contact in the complex and are poorly conserved among members of the Rho family (*28*). This region provides the core variability in primary sequence that therefore allows the selective pairing of individual Rho proteins with different GEF partners (*26*). Identification of the limit region in Dbl family GEFs is slightly more problematic as subtle hydrophobic and steric interactions determine whether a given DH domain acts

[1] Abbreviations: GDI, guanine nucleotide dissociation; GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein.

[2] Note that α helices $7_{a,b}$ from ref *23* are incorporated as α_5 in ref *26*.

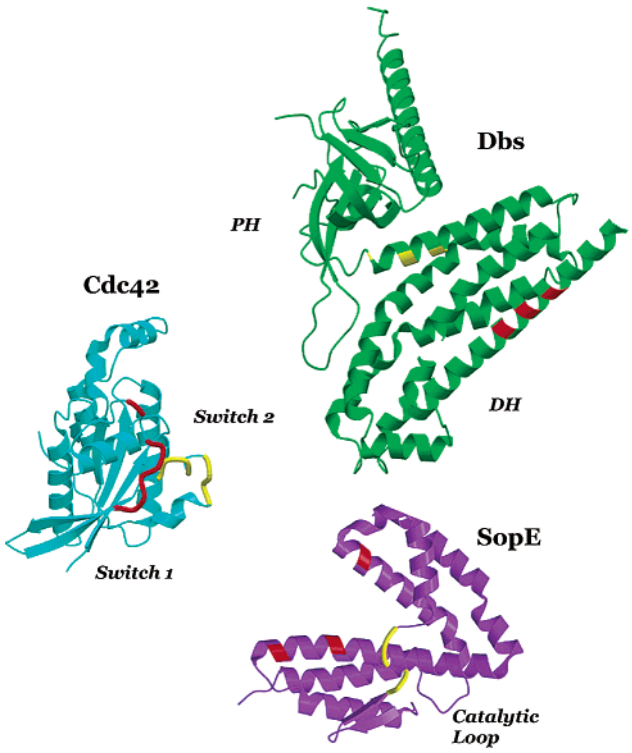

FIGURE 2: Molecular structures of dissimilar Cdc42 catalytic exchange domains. The Dbs structure is representative of the family of Dbl related proteins containing DH and PH domains. SopE is the catalytic Cdc42 exchange protein from *S. typhimurium*. Note that the structurally unrelated Cdc42 GEFs employ a similar mechanism of exchange via interactions with identical or nearby residues in Cdc42 switch regions. Primary switch interactions similar for the two GEFs are highlighted (red for switch 1 and yellow for switch 2). See refs *29* (Dbs-Cdc42) and *37* (SopE-Cdc42).

exclusively to catalyze GDP−GTP exchange on a single GTPase (e.g., Lbc for Rho) or if a DH domain exhibits a more promiscuous activity (e.g., Dbs activates both Rho and Cdc42 although these activities can be distinguished by mutation of Dbs; see ref *55*). Structural and biochemical data have so far assigned the major determinants of selectivity to the variable residues just N-terminal to and including α_5,[2] as these residues make complementary pairings with residues in the specificity patch of their cognate Rho GTPase (Figure 2; see ref *26* for a discussion of the predictive power of the residue pairing approach).

An additional interaction provided by the adjacent PH domain can, in some cases, contribute to the GEF activity profiles of Dbl family proteins (*29*). The orientation of this domain relative to the position of the DH domain is quite different in the available GEF structures. In some cases, the PH domain may primarily serve to localize GEF activity to a membrane surface, whereas in other cases (e.g., Dbs), it may directly contribute to the GEF activity (*29*). Specifically, the X-ray crystal structure of the complex of Dbs and Cdc42 reveals that the PH domain of Dbs swings on a short connecting loop which tethers it to the DH domain. The resulting angle of the PH domain relative to the DH domain in Dbs allows for additional contacts to be made with the switch 2 region in Cdc42 that are not seen in either of the closely related GEF−GTPase structures [i.e., Intersectin DHPH−Cdc42 (*26*) or Tiam1 DHPH−Rac1 (*27*)]. As it turns out, this additional contact that the PH domain of Dbs makes with residues Arg66 and His103 of Cdc42 provides only a minor contribution to the overall GEF activity. Rather, the critical contribution of the PH domain to GEF activity is made by a highly conserved tyrosine residue (889 in Dbs). This residue is thought to stabilize a catalytic triad (Tyr889 DbsPH, His814 DbsDH, and Asp65 of Cdc42) which, when disrupted in the Tyr889Phe or His814Ala mutant, results in the loss of GEF activity toward both Rho and Cdc42 (*29*).

The events that follow the initial recognition between the GTPase and the GEF domain lead to conformational changes in the GTPase which result in a decrease in its affinity for guanine nucleotide. In the present case of the Dbl family of GEFs, the recognition step is thought to involve complementary binding involving residues in the $\beta2-\beta3$ region of the GTPase to the variable $\alpha_4-\alpha_5$ helical region in the DH domain, resulting in a deformation of switch 2 (residues 59−67) in the GTPase. This in turn causes a coordinated intramolecular restructuring of the nucleotide-binding pocket where two critical changes in residue interactions occur: the insertion of the methyl side chain of Ala59 (Cdc42/Rac numbering; equivalent to 61 in Rho) into the coordination sphere of the Mg^{2+} ion and ion pairing of Lys16 with Glu62. The steric interference of Ala59 with the Mg^{2+} ion, coupled with the loss of the GDP β-phosphate interaction with Lys16, results in a dramatically lower binding affinity for guanine nucleotides. The induced structural changes that distort the nucleotide-binding pocket and lead to nucleotide dissociation, Mg^{2+} displacement, and "P-loop" disruption appear to be conserved in the mechanisms of guanine nucleotide exchange used by both Ras and Rho proteins (*30*).

Evolutionary Convergence of Rho GEF Function. The induced changes in the GDP-binding capability of Rho proteins by genetically unrelated GEFs are remarkably similar to those described above for DH family GEFs (*30*). For prokaryotes there are now several examples of molecular mimicry whereby protein functions have evolved that co-op the normal actin cytoskeletal remodeling processes of the host cell (*31*). The ability of *Listeria monocytogenes*, for example, to use the host cell Arp 2/3 cellular actin polymerization apparatus for intracellular motility has been studied in some detail (*32*). In addition, there are instances of bacterial protein mimicry and covalent modification of Rho GTPases that alter the regulation of the GTP-binding/GTP hydrolytic cycle in target G-proteins (*33*, *34*). The GEF-regulated remodeling of actin via Rho proteins is exploited in at least one well-documented case, the *Salmonella typhimurium* SopE protein (*35*). SopE has been shown to be a potent GEF for Cdc42 (*36*), although it shares no detectable sequence homology with Dbl family proteins. Recently, the X-ray crystal structure of the catalytic portion of SopE was solved in complex with Cdc42 and demonstrated an absence of structural homology as well, when compared with available Dbl protein structures (*37*). On the other hand, a comparison of the Cdc42−SopE and Cdc42−DHPH complexes, as well as comparisons of the structural changes that Cdc42 undergoes when complexed to these GEFs, suggests that similar coordinated changes occur in the residues affecting GDP binding (*37*; Figure 2). As in the case of DH domains, the SopE active site recognizes Cdc42 in the regions of switch 1 and switch 2 and locks Cdc42 into a conformation incompatible with high-affinity nucleotide binding (*37*). The primary contact amino acids in switch 2

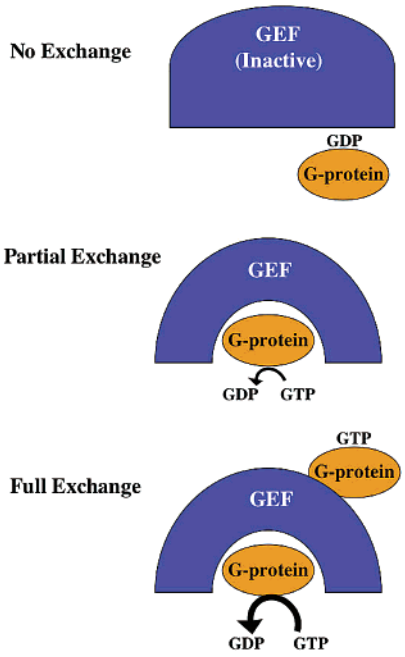

FIGURE 3: Model for allosteric regulation and positive cooperativity of exchange activity by GTPases. In this two-state activation model of GEF activity, reversal of the autoinhibited state leads to the intermediate level of exchange activity (as in Figure 1), which can be further increased by the binding of activated G-proteins to sites other than those responsible for exchange.

of Cdc42 that pair with SopE residues are Ala59, Gly60, and Gln61. Following the initial binding interaction in both DH domain-containing proteins and SopE, the affinity of the GTPase for guanine nucleotides is thought to be decreased after a peptide "flip" in switch 2 results in the repositioning of the methyl group of Ala59 (in Cdc42) to within 2 Å of the Mg^{2+} binding site. Described as a push (of switch 1)−pull (of switch 2) mechanism (*30*), the conformational changes surrounding the bound nucleotide result in the approach of the hydrophobic side chain of Ala59 to the Mg^{2+} coordination sphere. As in the case of DH-induced changes, an unfavorable steric interaction between Mg^{2+} and the methyl group of Ala59 induced by SopE binding results in the destabilization of Mg^{2+} binding with a consequent lower binding affinity for the nucleotide. Additional residues in the SopE/Cdc42 structure making contributions to weaker nucleotide-binding affinity overlay exactly with the cognate Rac1 residues observed in the Tiam-1/Rac crystal structure (e.g., Phe28 and Phe37; see Figure 3 in ref *37*), further supporting the view that SopE and Dbl share similar molecular mechanisms of nucleotide exchange.

Structural and biochemical information for other GEF interactions with small G-protein indicate that the region of switch 2 is used for initial protein coupling. A similar deformation of this region occurs in the case of the Ras/Sos complex, resulting in the movement of the highly conserved Ala59 residue into close proximity with the coordinated Mg^{2+} ion in the nucleotide-binding pocket. Steric interference of the Mg^{2+} coordination sphere by Ala59 in Ras is important in loosening the grip of the GTPase for nucleotide in the Ras/Sos complex as well (*30*), supporting the view that the general mechanistic features of GEF-catalyzed GDP dissociation from Rho family proteins are conserved for Ras family proteins and their GEFs.

Another genetically distinct group of proteins of eukaryotic origin that have evolved the ability to act as GEFs for Rho GTPases are members of the DOCK180/CED-5/Myoblast City (mammalian/worm/fly) family (*38*). Currently, there are approximately 10 mammalian Dock180-related proteins with binding specificity and/or GEF activity toward Rac or Cdc42 (*39*). In mammalian cells, these proteins play critical roles in cell migration, phagocytosis, and cytoskeletal remodeling (*40*), while their counterparts in *Caenorhabditis elegans* and *Drosophila* mediate developmental processes that require proper temporal and spatial control of cytoskeletal organization (*41*, *42*). All members of this family possess a "Docker" (*39*) or *d*ock *h*omology *r*egion 2 (DHR-2) domain which consists of approximately 500−550 residues. The isolated, recombinant DHR-2 domain appears in some studies to be sufficient to catalyze the GDP−GTP exchange reaction of Rho proteins *in vitro* (*39*, *43*). In the case of DHR-2 from Dock-2, a close homologue of Dock180 (the current nomenclature designates Dock180 as Dock-1; see refs *39* and *44*), it has been suggested that the DHR-2 domain may be structurally similar to the tandem DH−PH domains found in Dbl proteins (*39*). Questions still remain regarding the limit of the functional GEF domain or protein complex that gives rise to the observed *in vitro* activity (*44*). Specifically, it has been shown for the case of the DOCK180-limit GEF domain that the accessory protein ELMO/CED12 (for engulfment and motility) is necessary for DOCK180 to stimulate GDP−GTP exchange (*45*). This model proposes that the Docker domain of Dock180 is able to bind nucleotide-free Rac but that GDP−GTP exchange can only proceed when Elmo is bound to the N-terminal region of Dock180 (*45*). In this view, the complex of Elmo and Dock180 acts in concert to provide a binding site for Rac (i.e., Docker) and a catalytic GEF activity (ELMO). Since other closely related Docker domains from Dock2 and Dock9/Zizimin reportedly have intrinsic GEF activity without the need for additional factors (*39*, *43*), the DHR-2 domain in some cases may be sufficient for catalyzing guanine nucleotide exchange. Further characterization of the individual Dock isoforms may help to delineate the minimal requirements for nucleotide exchange and reveal cofactors necessary for GEF activity such as have been shown for the Dbl family of proteins (*46*, *47*). It is in either case apparent that the Dock180/CED-5/MBC superfamily represents a conserved and distinct evolutionary path to regulating Rho GTPases. An appreciation of the important mechanistic similarities and differences that may exist between the conventional DH−PH domain-containing proteins and the Docker family awaits the availability of structural data for the latter.

Allosteric Regulation of GEF−GTPase Exchange by Activated GTPases. As an example of a specific subset of regulatory protein−protein interactions, there is some evidence that DH−PH domains may serve as direct links in signal transduction pathways by providing target sites that lie outside of the cleft used to catalyze GDP−GTP exchange (Figure 3). In the case of the Rho-specific GEF p115/Lsc (*47*, *48*), nucleotide exchange activity is dependent on the upstream activation of the heterotrimeric G-protein α_{13} subunit (*47*, *49*). The active conformation of α_{13} recognizes two sites on full-length p115/Lsc. One site is the rgRGS domain at the p115/Lsc N-terminus, which facilitates GTP

hydrolysis in α_{13} and, presumably through intramolecular communication between the rgRGS and DH domains, increases nucleotide exchange activity by p115/Lsc for RhoA (*50*). Deletion analysis of p115/Lsc identified a second binding site, mapping to a region of the DH domain and dependent on AlF_4^- activation of α_{13} for tight binding (*51*). It will be interesting to see if the two-site binding of p115/Lsc to active α_{13} bears any similarity to the interactions seen in the crystal stucture complex of α_t/RGS4/γPDE, as the authors speculate (*50*). In this scenario, the α_{13}-p115/Lsc DH domain would bind to switch 2 in activated α_{13}, in a manner analogous to interactions observed in the α_t/α_i chimerae/γPDE/RGS4 crystal structure (*51*). Given the highly conserved structures observed for DH domains, perhaps the switch 2 region in other heterotrimeric G-protein α subunits targets a heretofore unrecognized common fold of the DH domain structure, away from the active site cleft that stimulates GDP−GTP exchange, and in this way contributes to the observed level of GEF activity.

In fact, it has recently been demonstrated that Ras itself, when activated by the Ras GEF Sos and bound to GTP, can enhance the GEF activity of Sos by binding to a distinct site on the Sos molecule (*52*). As the recently reported crystal structure shows, this positive cooperativity is mediated by an interaction between GTP-bound Ras and Sos that is comparable in its structural details to the binding of Ras to its target/effector, phosphoinositide 3-kinase (*52*). As this study suggests, positive feedback mediated by activated GTP-bound Ras molecules might greatly enhance the localized effect of Sos activity, concentrating the active pool of Ras at the inner membrane site of receptor clustering. Due to the absence of structural homology between GEFs specific for Ras and Rho GTPases, it is difficult to draw conclusions from Sos that may apply to the Dbl family or other Rho GEFs. It is interesting to note, however, that possible allosteric interactions governing the GEF activity of DH domain-containing proteins and mediated by active and inactive states of GTPases have been observed for the Cool/Pix proteins (Q. Feng and D. Baird, personal communication). Consistent with this emerging theme of GEF allosteric regulation by active forms of GTPases, a recent study documents the importance of active RhoG binding to the Dock180/Elmo complex and stimulating Dock180 exchange activity toward Rac (*60*).

Direct binding and control of DH−PH domains by members of the Rho family may offer one avenue for *in vivo* cross talk and feedback among these GTPases and their activators. In the future, it will be interesting to see how often this mode of GTPase regulation is employed in the governance of cell signaling pathways.

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