J. Biochem. **120**, 215-228 (1996)

The Small GTPase Rho: Cellular Functions and Signal Transduction

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Received for publication, May 10, 1996

Rho, a Ras homologue of small GTPase, is present from yeast to mammals. It shuttles between the active GTP-bound form and the inactive GDP-bound form and works as a switch in stimulus-evoked cell adhesion and motility, enhancement of contractile responses, and cytokinesis. In these actions, Rho directs the reorganization of the actin cytoskeleton at a specific time and at a specific site in the cell. It also activates serum response factor possibly *via* a kinase cascade and mediates a growth signal to nuclei. Two signalling processes are known to lead to Rho activation: one is activation of certain types of G-protein-coupled receptors such as lysophosphatidic acid receptor, and the other is activation of other small GTPases including Ras, CDC42, and Rac. Molecules catalyzing the GDP-GTP exchange of Rho, Rho guanine nucleotide exchange factors (Rho GEF), and those catalyzing the acceleration of GTP hydrolysis, Rho GTPase activating proteins (Rho GAP), were identified as Dbl- and Bcr-containing molecules, respectively. In addition, a molecule inhibiting guanine nucleotide exchange of Rho, Rho guanine nucleotide dissociation inhibitor (Rho-GDI), was isolated and characterized. More recently, putative Rho targets possibly mediating various Rho actions have been identified by their selective interaction with GTP-bound Rho. They include lipid kinases such as phosphatidyl-inositol-5-kinase and protein serine/threonine kinases such as PKN and p160ROCK. A model of the molecular mechanism of action of Rho constructed on the basis of these findings is presented. There are, however, still many unclarified links between cell stimulation, Rho activation and final Rho actions.

Key words: actin cytoskeleton, *botulinum* C3 exoenzyme, cell adhesion , GTPase, Rho.

More than 50 Ras-related small GTPases are now known to be present in mammalian cells and yeast. Among them, Rho was the first Ras homologue identified in 1985 as a mammalian gene homologous to *ras*, from which was derived its name (Ras homologue) (*1*). Awareness that Ras homologues of small GTPases constitute a family of signaling molecules working in a variety of cell functions arose in the mid-80s from genetic studies in yeast and from analyses of the effects of GTP on various mammalian cell functions. This awareness stimulated a biochemical as well as molecular biological search for these proteins, leading to the purification of several small GTPases including Rho from tissue homogenates (*2, 3*) and to the discovery of Rho relatives, Rac and Cdc42Hs, among other Ras homologues (*4-6*). During the same period, a novel ADP-ribosyltransferase was found in *Clostridium botulinum* that specifically ADP-ribosylates a 22 kDa protein in mammalian cells in a guanine nucleotide-dependent manner (*7, 8*). The substrate protein for this enzyme, now known as *botulinum* C3 exoenzyme, was purified and identified as a Rho protein (*9, 10*). Because this ADP-ribosylation occurs at Asn in the putative effector binding domain of Rho, it was suggested to block the signal transduction of Rho (*11*). This hypothesis was later verified by comparison of a cell phenotype induced by C3 exoenzyme with that induced by activated Rho. These studies revealed that Rho had a role in the stimulus-evoked reorganization of certain types of actin cytoskeleton. Rac and Cdc42 were then found to induce reorganization of similar but different types of actin cytoskeleton. Through these analyses, it has been hypothesized that these Rho GTPases are activated in sequence in the cell and regulate dynamic movement of the cell. In this article, cellular Rho functions revealed by these studies are reviewed, and biochemical mechanisms responsible for these actions of Rho are discussed. Possible roles for recently identified putative Rho effectors in these transduction processes are also discussed.

Cellular Rho functions

The mammalian Rho family thus far comprises three isoforms of Rho (A, B, and C), two isoforms of Rac (1 and 2), and one isoform each of Cdc42, RhoG, and TC10, the last two being distant members of this family. Among the three isoforms of Rho, RhoA is most ubiquitously and abundantly expressed in the body, and has been most extensively studied. The cellular Rho functions described below are, therefore, mostly related to RhoA. Whether RhoB and C serve redundant roles or have distinct functions remains unknown. Expression of Rho A, B, and C as an epitope-tagged protein and immunocytochemistry using an anti-epitope antibody showed different intracellular localization of each member (*12*). It was also reported that RhoB is an immediate early gene induced by growth factors such as EGF and PDGF, non-receptor tyrosine kinases such as v-Src and v-Fps, UV irradiation, or DNA damaging drugs such as cisplatin and *N*-methyl-*N*-nitrosourea (*13, 14*).

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In this section, experimental findings indicating the involvement of Rho in cell adhesion, motility, cytokinesis, contractile responses, and cell growth are summarized. Its role in the whole body is discussed based on *in vitro* findings in cultured cells. Particular attention is paid to findings suggesting a molecular mode of action.

Stimulus-induced cell adhesion and motility. The first indication that Rho is involved in the organization of the actin cytoskeleton was obtained by an experiment using C3 exoenzyme in cultured Vero cells, which resulted in cell rounding and dissolution of actin fibers in the cells (*15*). At that time, however, the siginificance of the result was not clear. Neither was it whether it was really due to ADP-ribosylation of Rho, or, if so, whether this reaction made Rho active or inactive. By the analogy to a constitutively active Val^{12}-Ras mutation, Paterson *et al.* (*16*) introduced a Val mutation into Gly^{14} of human RhoA. This mutation rendered Rho a constitutively active GTP-bound form, and this mutant protein was microinjected into Swiss 3T3 cells. In minutes, the cell bodies of injected cells contracted and several finger-like processes were extended at the periphery of the cells. Actin staining with phalloidin revealed that strong actin stress fibers originated from the processes and extended across the cell. This appeared to be a very different morphology to that induced by C3 exoenzyme treatment of these cells; round cell bodies and loss of stress fibers were noted. Stress fibers are formed between cell structures called "focal adhesions" or "focal contacts." Focal adhesions consist of integrins and cytoplasmic proteins such as vinculin and talin which form a multi-protein complex binding to the cytoplasmic tail of integrin molecules on the ventral surface of cells. This structure is known to link cells to substratum *via* integrin binding to extracellular matrix (ECM) protein. Although not explicitly stated in this report, the above results indicated that Rho works to mediate cell adhesion to the substratum, and in view of the GTPase nature of Rho, it was likely to play a switching role in processes evoked by external stimuli. This hypothesis was tested by two groups. Ridley and Hall (*17*), staining Swiss 3T3 cells with phalloidin and anti-vinculin antibody, found that focal adhesions and stress fibers were induced by serum in these cells. They also found that inactivation of Rho by C3 exoenzyme blocked this serum action and that microinjection of activated Rho, on the other hand, mimicked the serum response. Moreover, they found that the major serum factor responsible for this effect was lysophosphatidic acid (LPA). Using C3 exoenzyme, we independently examined similar actions of Rho in other cell systems, *i.e.* blood platelets and lymphocytes (*18, 19*), because both types of cells show stimulus-evoked, integrin-mediated cell adhesions. Stimuli such as thrombin activate platelets and cause platelet aggregation, an adhesion reaction mediated by platelet integrin, GPIIb/IIIa, binding to fibrinogen. Lymphocytes show a stimulus-induced adhesion to endothelial cells mediated by leukocyte integrin, LFA-1, and ICAM on endothelial cells. Inactivation of Rho with C3 exoenzyme blocked adhesion of both types of cells, although it had no effect on second messenger generation and integrin expression on the cell surface. Because integrin molecules present on the cell surface are activated on cell stimulation to bind their ligands, these results suggest that Rho receives a signal downstream of the second messenger and converts it to activate integrin for cell adhesion.

Two pathways have been proposed for integrin activation. One is inside-out signalling, in which stimuli such as growth factor signals are transmitted inside the cell, then activating integrin. The other is outside-in signalling, in which ligation of integrin with ECM itself activates integrin and triggers signalling events based on the formation of focal adhesion structures (see Ref. *20* for review). The latter pathway was proposed based on the observations that serum-starved cells (cells without any extracellular stimuli) can attach to and extend on a dish coated with ECM proteins and that these cells can transmit growth signals inside. Based on these results, there has been disagreement on which of the two pathways is of primary importance in Rho activation and cell adhesion. Hotchin and Hall (*21*) recently examined this issue by plating serum-starved Swiss 3T3 cells on dishes coated with either poly-L-lysine or fibronectin in the presence or absence of serum. Their results indicate that at least in their cells, both serum stimulation and ligation with ECM protein are essential in the formation of focal adhesions and stress fibers, *i.e.* cell adhesion to the substratum. It is known that the effect of serum starvation on the loss of stress fibers, *i.e.* Rho inactivation, is quite variable in cultured cells, and can vary from subclone to subclone even in the same cell line. The above discrepancy, therefore, probably resulted from comparing cells with different sensitivities to serum starvation.

The above findings on the actions of Rho on cell morphology led Hall and his collaborators to further examine the actions of Rho relatives, Rac and Cdc42, by microinjecting a constitutively active form of each of the proteins to follow phenotypic changes. They found that each protein caused different morphological changes in cells by inducing the formation of different types of actin cytoskeleton; Rac induces lamellipodia with membrane actin ruffles and Cdc42 induces filopodia with actin microspikes (*22, 23*). A similar finding with the Cdc 42 phenotype was reported by Kozma *et al.* (*24*). Interestingly, when cells showing the Rac- or Cdc42-phenotype were stained with antibodies against known components of focal adhesions such as vinculin, punctate staining was found in association with each of these actin cytoskeletons, on the base of Rac-induced membrane ruffles and at the bottom of CDC42-induced microspikes. Thus, like Rho, these GTPases appear to induce multi-protein complexes at specific site in the cells to organize specific types of actin cytoskeleton, which Hall *et al.* called "focal complexes." Studies using dominant negative mutants of these GTPases revealed that, like LPA for Rho activation, certain growth factors can selectively induce the above morphological changes *via* the activation of these GTPases, *e.g.* PDGF for Rac activation and membrane ruffles and bradykinin for Cdc42 activation and filopodia. What is more intriguing is the observation that the GTPase-induced morphological changes occur in a definite sequence in the cell, *i.e.* Cdc42-induced filopodia followed by Rac-dependent membrane ruffles and then Rho-dependent stress fiber formation. This observation led to the hypothesis that this sequential activation of Rho GTPases (GTPase cascade), Cdc42→Rac→Rho, may drive cell movement (*23*). If so, concerted action of three Rho GTPases is required for cell motility. These actions are depicted in Fig. 1A. Microinjection studies also found that activated Ras can induce Rac-dependent membrane ruffles

and then Rho-dependent stress fiber formation, indicating the presence of another cascade of Ras→Rac→Rho GTPases. This work led to studies examining the roles of Rac and Rho in Ras-induced cell growth and transformation (see below).

The above findings naturally stimulated research to examine the roles of the Rho GTPases in cell motility. Indeed, several groups identified an role for Rho GTPases, but which Rho GTPase is essential remains controversial. Takai and collaborators used both C3 exoenzyme (which selectively inhibits Rho action) and Rho-GDI (which inhibits both Rho and Rac actions) to differentiate the actions of Rho and Rac, and found in cultured KB cells that membrane ruffling induced by insulin was due to Rac action, whereas that induced by hepatocyte growth factor (HGF) or TPA was dependent on Rho (*25*). They also analyzed the motility of cultured mouse 308R keratinocytes induced by HGF and anti-cadherin antibodies. They determined the effects of C3 and Rho-GDI on HGF-induced cell spreading and scattering and again found Rho-dependence (*26*). What was more interesting was their observation that this cell motility was not inhibited by dominant negative Rac. They further extended their observation to MDCK cells stably expressing myc-tagged RhoA and reported Rho was translocated from the cytosol to membrane ruffle area by stimulation with TPA (*27*). On the other hand, Hall and his collaborators also using HGF-stimulated MDCK cells found that HGF-induced motility was suppressed by dominant negative N^{17}-Rac or Y13-259 monoclonal anti-Ras antibody, suggesting that this motility was induced by the Ras →Rac pathway (*28*). Their experiment with C3 exoenzyme showed dual effects as that 2 μg/ml microinjection suppressed stress fibers but not motility, whereas 20 μg/ml microinjection inhibited both stress fiber and motility. In contrast, microinjection of dominant active Val^{14}-Rho inhibited the motility. We do not know at present how to explain this discrepancy. The two groups may have examined different cell functions required for cell motility, because they used similar but different protocols. It is interesting that both groups found that cell motility could not be induced by microinjecting the GTP-form of either Rho or Rac alone, suggesting that both are essential but not sufficient alone.

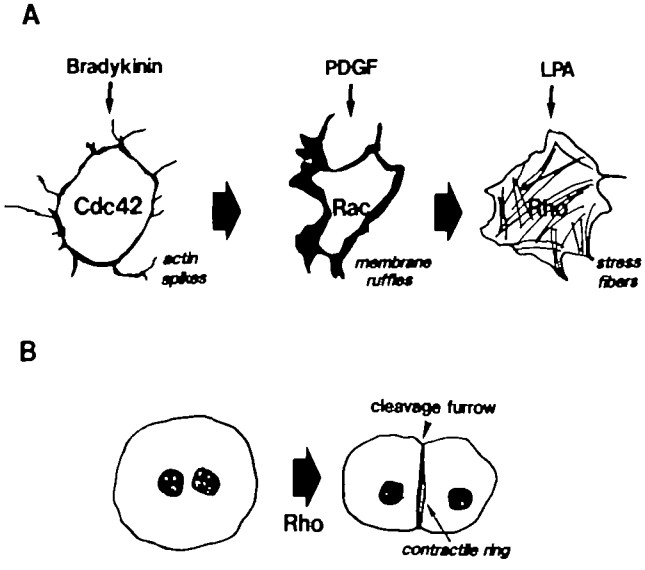

Fig. 1. **A: Changes in cell morphology and the actin cytoskeleton induced by the Rho GTPase cascade.** Each of the Rho family of GTPases, Cdc42, Rac, and Rho, is activated by a specific extracellular signal: bradykinin, platelet-derived growth factor (PDGF), and lysophosphatidic acid (LPA), respectively, and directs the organization of a specific type of actin cytoskeleton to induce the characteristic morphological changes. Furthermore, these GTPases are activated in the sequence of Cdc42→Rac→Rho in the cell. This sequence is associated with the time-dependent change in cell morphology from filopodia to lamellipodia to stress fibers. **B: Involvement of Rho in cytokinesis.** Rho is activated during M-phase following nuclear division and induces the formation of a contractile ring in the cleavage furrow. Rho action is required throughout cytokinesis, because inactivation would cause the loss of the preexisting contractile ring.

The above results showed that all of the Rho family of GTPases equally induce actin cytoskeleton organization but each at different location in the cells. This may suggest that these GTPases have two functions; one is to induce actin polymerization and the other is to organize the focal complexes at certain location in the cell, the latter being specific to each member. This may further suggest that these two functions are exerted separately by Rho proteins. Indeed, inhibition of actin polymerization by cytochalasin did not interfere with the induction of Rho-dependent adhesion of lymphocytes (*19*), indicating that actin polymerization *per se* is not a prerequisite for integrin activation. Furthermore, microinjection of activated Rho into cells plated on a poly-L-lysine-coated dish induced the formation of randomly swirling actin fibers in cells without focal adhesion formation (*21*). These results are consistent with the hypothesis above and suggest that each of the Rho family GTPases activates multiple target proteins. At least one of these proteins mediate actin polymerization and is in common to all members. It is tempting to speculate that this family of GTPases may have multiple domains, each interacting with a different effector. Of particular interest in this respect is a recent paper by Diekmann *et al.* (*29*). They generated various Rac/Rho chimeras and analyzed their activity by microinjecting them into Swiss 3T3 cells. One of them, $Rac^{73}Rho$ (73 N-terminal residues from Rac, the remainder from Rho) induced the formation of the Rho phenotype of stress fibers and focal adhesions. This result indicated that specificity of action was determined by the C-terminal part of the molecule. This may further indicate that the N-terminal 73 residues including the so-called effector loop may direct actin polymerization, a function common to Rho family proteins. If so, the opposite $Rho^{73}Rac$ chimera may induce the formation of membrane ruffles and not stress fibers. At present, there is no report on such a chimera.

Cytokinesis. Early in one of the initial studies using C3 exoenzyme it was found that the incubation of cultured cells with this enzyme led to the appearance of mutilnucleate giant cells (*30*). However, probably due to the concomitant G1 block of cell cycle progression (see below), the population of multinucleate giant cells was quite low (only a few percent of the total), and it was not known whether Rho plays a crucial role in cell division. This issue was later examined more directly in fertilized eggs of *Xenopus laevis* (*31*) and sea urchin (*32*). When fertilized eggs of either species received microinjection of either C3 exoenzyme or Rho-GDI, cytokinesis following nuclear

division did not occur or the ongoing cytokinesis was aborted with the disappearance of the cleavage furrow. Nuclear division, nonetheless, continued, resulting in multinucleate eggs sometimes containing 8 or 16 nuclei. These findings indicate that Rho has a crucial role in cytokinesis, receiving a signal of completion of nuclear division to induce and maintain cytokinesis, and that inactivation of Rho completely uncouples nuclear division from cell division. Cytokinesis is carried out by the contraction of the actin "contractile ring" formed in the cleavage furrow in the middle of the cell body. When treated eggs were stained for actin with phalloidin, this ring was found to be absent, while the fertilization-induced cortical actin meshwork remained intact in the treated cells. These results suggest that Rho works as a switch between nuclear and cell division by the induction and maintenance of this specific type of actin cytoskeleton (Fig. 1B). They also indicate that Rho induces formation of different types of actin cytoskeleton at different phases of the cell cycle; stress fibers in the G1 phase to flatten the cells and the contractile ring to perform cytokinesis of cells at the M phase. Because cells become round upon entering the M phase, the Rho effect on stress fibers should be terminated, and a switch made to Rho functions for cytokinesis. How this switching occurs remains unknown.

Smooth muscle contraction and other contractile responses. Smooth muscle contraction, like skeletal muscle contraction, is triggered by a rise in the intracellular free calcium concentration. This calcium rise in this tissue leads to the activation of calmodulin-dependent myosin light chain kinase, which phosphorylates myosin light chain to induce contraction. The extent of contraction is determined primarily by the intracellular concentration of free calcium ions. However, it has been known for some time that the extent of contraction differs depending on the agonist at the same intracellular free calcium ion concentration. It was hypothesized that agonists induce, in addition to a calcium rise, a parallel unknown signal which makes the smooth muscle more sensitive to calcium. The GTP-dependency of this phenomenon, known as "calcium sensitivity" (*33*), became evident when GTP added to permeabilized smooth muscle was shown to induce additional contraction at a fixed concentration of free calcium. During identification of small GTP-binding proteins in various tissues, Takai and collaborators found that Rho and Rap are highly expressed in aortic smooth muscle cells. To test their roles, they made permeabilized preparations of mesenteric artery and examined the effects of C3 exoenzyme treatment on the GTP-induced calcium sensitivity of contraction (*34*). The enzyme treatment completely abolished the increase in the sensitivity, which was then restored by the addition of GTPγS-bound RhoA to the preparation. This was the first report suggesting the involvement of Rho in the calcium sensitivity process, but its mechanism remained unknown until in 1994 another Japanese group examined the effect of Rho on MLC phosphorylation (*35*). Using the mobility shift of phosphorylated MLC as a phosphorylation parameter, they found that activated Rho indeed increased the amount of phosphorylated MLC, and that it was not due to the increase in the phosphorylation rate but to the decrease in the dephosphorylation reaction. A possible mechanism for this Rho action is depicted in Fig. 2. A more recent report (*36*) indicates that the phosphorylation of the p130 regulatory subunit of MLC phosphatase is involved in the regulation of phosphatase activity (see below).

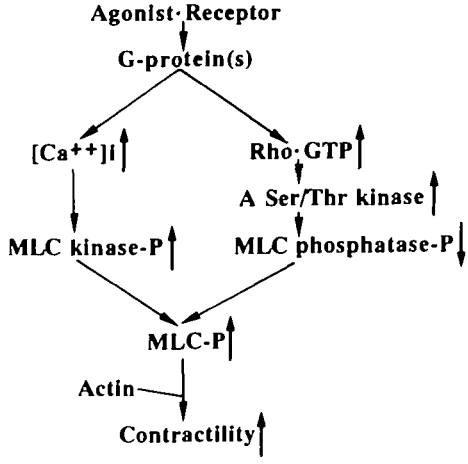

Fig. 2. **Dual control of smooth muscle contractility and regulation of myosin light chain phosphatase activity by Rho.** Most agonists induce both a rise in the intracellular free calcium concentration and the activation of Rho, although the relative effects may vary among the different agonists. The rise in the calcium ion level activates MLC kinase to increase MLC phosphorylation. Activated Rho appears to inhibit MLC phosphatase activity *via* a serine/threonine kinase, which also contributes to the increase in MLC phosphorylation.

The above findings suggest that Rho may be involved in the enhancement of contractile responses in other types of cells. In retrospect, this was already evident in the first report on the effect of microinjection of activated Rho, which showed cell contraction in addition to strong stress fiber formation (*16*). Indeed, cell contraction is frequently seen when the activated form of Rho is expressed or injected in cultured cells. Rho-dependent contraction was most clearly found in cultured neuronal cells. N1E-115 neuroblastoma cells, NG108-15 glioma×neuroblastoma hybrid cells, and PC-12 pheochromocytoma cells extend long neurites in response to nerve growth factor or serum starvation. When either thrombin or LPA was added to these cells, the neurites quickly retracted and the cells became round. This is apparently a contractile response caused by tension produced by the actomyosin system (*37*). When neurites were pretreated with C3 exoenzyme and then exposed to the above agents, they no longer showed retraction, indicating that neurite retraction was a process dependent on Rho activation. It is known that cultured neuroblastoma or PC12 pheochromocytoma cells show round cell bodies in the presence of serum and extend neurites upon serum starvation. The above results indicate that this is due to serum-induced activation of Rho in the cells. Consistently, C3 exoenzyme treatment induced a number of long neurites in these cells (*37, 38*).

Cell growth, cell transformation, and nuclear signalling. Avraham and Weinberg (*39*) examined the effects of transfection of *rhoA* and found that cells bearing multiple copies of transfected *rhoA* showed an increase in both the growth rate and saturation density. These results were consistent with experiments using C3 exoenzyme in cultured PC-12 cells, in which ribosylation of Rho caused

inhibition of cell proliferation (*38*). The growth inhibitory effect of C3 exoenzyme treatment was further examined in Swiss 3T3 cells (*40*). This study showed that C3 exoenzyme-treated cells accumulated in the G_1 phase of the cell cycle, indicating that Rho is involved in G_1-S progression. However, the results of this study were discussed only in relation to Rho-induced cell adhesion to the substratum and in the context of the anchorage-dependency of normal cell growth. The relationship between Rho and other cell growth signalling was not examined until quite recently. Interest in the growth promoting activity of the Rho family proteins has been revived by the identification of a family of potential GDP/GTP exchange factors for Rho such as Dbl, Ost, and Lbc as oncogenes (see below), and by the finding described previously that Ras can activate Rac and Rho in sequence (*22*). Two groups have examined the possible involvement of Rac and Rho in Ras-induced cell growth and transformation (*41, 42*). Both found that Ras transformation could be at least partially blocked by co- transfection with either dominant negative Rac or dominant negative Rho, and that activated Rac or Rho induced cell transformation synergistically with the Raf-MAPK pathway. Their findings were consistent with a model whereby activated Ras simultaneously stimulates the two pathways to induce cell proliferation and transformation. Interestingly, cells transformed by co-transfection with activated Raf and Rho showed highly refractile, poorly adherent cell morphology, which appears to be quite different from the so-called Rho phenotype showing highly adherent cells with strong stress fibers. This finding suggests that the growth promoting action of Rho is mediated by a pathway separate from that leading to cell adhesion, the latter being downregulated by the former in conjunction with Ras activation. Then, how does Rho or Rac transmit growth signals synergistically with Ras? Such synergism may occur in nuclei. Hill and Treisman (*43*) studied the role of Rho family proteins in serum-induced c-fos transcription. This response involves serum response element (SRE) interaction with the two transcription factors SRF (serum response factor) and TCF (ternary complex factor). They found that the Rho family proteins, Rho, Rac, and Cdc42, can induce activation of SRF (and SRF-dependent SRE activation), and that Rho indeed mediates this activation in response to several stimuli including serum and LPA. Because activations of TCF and SRF synergistically induce c-fos transcription and because Ras has been shown to activate TCF *via* the Raf-MAPK pathway, these results suggest that the Ras and Rho pathways converge at nuclei to enhance this transcription synergistically. By analogy with a yeast pheromone pathway, these authors suspected the presence of a kinase cascade for the Rho-mediated nuclear pathway. However, they did not find any correlation between SRF-dependent SRE activation and activation of known kinases such as MEK, SAPK/JNK, and MPK/p38. On the other hand, Coso *et al.* (*44*) and Minden *et al.* (*45*) found that Rac and Cdc42 but not Rho can activate the MEKK-SEK/MKK4-JNK/SAPK kinase cascade to enhance c-Jun transcription activity, mediating the effect of TNF-α, EGF, and v-src. Recent reports indicate that Rac and Cdc42 do not directly act on MEKK1 but act first on PAK (*46*), a Rac/Cdc42 target, which in turn activates the MEKK-JNK pathway (*47*).

Roles of Rho GTPases in the body. Perhaps because Rho is involved in functions essential to the cell such as cytokinesis, and also because redundancy is expected among the three Rho proteins (A, B, and C), there have been no gene-targeting studies of these genes. However, studies on cultured cells and the *in vitro* model systems indicate roles for Rho in haemostasis (*18*), inflammatory cell migration (*19, 48*), and tumor cell invasion and metastasis (*49-51*). The last is of interest since Rac and Rho are downstream of one of the Ras signalling pathways. Tumor cell metastasis and invasion is a complex series of events including dissociation from the original tissues, degradation of the surrounding matrix, adhesion and migration through the cell layer (transcytosis). The cadherin/catenin system and matrix metalloproteases are thought to be involved in the first two events, and involvement of the Rho family proteins is suspected in the last. Tumor cell transcytosis can be studied *in vitro* by adding tumor cells on a monolayer of mesothelial or endothelial cells. The tumor cells move through the monolayer and make focal colonies underneath. The invasive potential can be quantified by counting the number of colonies they form. Using this method, Imamura *et al.* (*49*) found that rat hepatoma MM1 cells could transmigrate the mesothelium monolayer and that this migration requires serum or LPA. When they treated MM1 cells with C3 exoenzyme, they did not do transcytosis even in the presence of LPA. Based on these results, they concluded that activation of Rho is required for transmigration of MM1 cells. Interestingly, when they screened several cell lines, there was a difference in the requirement for serum/LPA among cells, and serum-dependent cells became independent when they were stably transformed with Val^{14}-RhoA (*50*). These results indicates the possible importance of Rho in tumor invasion *in vivo*. Using a similar assay, John Collard and collaborators screened genes which, when transfected, imparted transmigration capacity to cultured lymphoma cells. By this approach, they isolated a gene which they called Tiam-1 (T-lymphocyte invasion associated molecule) (*51*). This gene contains a region homologous to the known sequence of Rho GEF (see below). When they transfected Tiam-1 to cultured fibroblasts, they saw membrane ruffles which could be suppressed by dominant negative Rac (*52*). These results indicate that Rac is a rate limiting factor for transcytosis in this type of cell. As discussed above, because motility in different cells had different prerequisites, the above two results probably reflect properties of each cell line and the concerted actions of both Rho and Rac appear essential.

Because cell adhesion and migration are essential steps in development, it is quite likely that Rho GTPases are involved in the organization of tissue architecture and organogenesis. Direct proof is still lacking, but circumstantial evidences continues to accumulate. For example, expression of dominant active Rac under an L7 promotor in cerebellar Purkinje cells resulted in a drastic reduction of axon extension and the appearance of abnormally small and numerous dendritic spines (*53*). This study using dominant positive Rac may indicate that switching on and off of Rho/Rac activities should be accurately controlled during development. Such a phenomenon was also observed when *Drosophila* Rho1 was expressed in the eye during development. While the expression level was rather low, about an amount equal to the level of endogenous Rho, it resulted in

the loss of secondary and tertiary pigment cells as well as a reduction in the number of photoreceptor cells (*54*). The involvement of Rho in organogenesis has also been suggested by analyses of genetic diseases. One of the analyses was carried out on faciogenital dysplasia (FGDY, Aarskog-Scott syndrome) (*55*). This is an X-linked developmental disorder characterized by a disproportionately short stature and by facial, skeletal, and urogenital anomalies. A candidate gene for FGDY (FGD1) was identified and characterized. It encodes a 961 amino acid protein, a part of which shows a strong homology to Dbl, a characteristic motif of Rho/Rac GEFs. FGD1 is disrupted by a breakpoint in FGDY patients and FGD1 mutations co-segregate with the disease. These results indicate that FGD1 is responsible for FGDY and suggest that FGD1, a putative Rho/Rac GEF, is involved in mammalian development. Another link between a hereditary disorder and the defect in signalling of Rho family GTPases has been found in Wiskott-Aldrich syndrome (WAS) and Cdc42. This syndrome is a recessive X-linked immunodeficiency disorder characterized by abnormalities in T and B cell functions, thrombocytopenia, and a high susceptibility to infections. The affected gene product was identified by positional cloning and called WASP. Recent two hybrid screening (*56*) and identification of a Cdc42-binding protein (*57*) demonstrated a physical association of WASP and Cdc42 in a GTP-dependent manner. It was also found that the Cdc42 binding domain of WASP shares the binding motif conserved in many putative Rac/Cdc42 target proteins. Because lymphocytes in WAS show abnormal cell surface morphology, weak chemotactic activity and reduced proliferative capacity, WASP may work as a downstream target of Cdc42 to mediate effects on cell surface structure and cell proliferation.

Signal transduction of Rho

Upstream signalling. Signalling pathways involving Rho were identified in cultured cells using C3 exoenzyme, the inhibition of certain cell functions by this enzyme treatment being taken as evidence for Rho involvement. These studies have identified many external stimuli as Rho activating signals. They include serum, LPA, thrombin, endothelin, and bombesin (*17, 37, 58*). All of these substances act on a cell surface receptor coupled to a heterotrimeric G-protein(s). Thus, the heterotrimeric G-protein-mediated pathway appears to be the major upstream pathway for Rho activation. The receptors for the above substances usually couple to multiple G-proteins to exert a variety of actions. For example, LPA receptor couples to G_i, G_q, and perhaps others (*59*). G_i is known to mediate LPA action to activate Ras and cell growth. Inhibition of G_i by pertussis toxin inhibited growth stimulation by LPA but not stress fiber induction, indicating that stress fiber formation (*i.e.* Rho activation) is mediated by G-proteins different from G_i. This issue was directly addressed by Buhl *et al.* (*60*) who microinjected various constitutively activated G_α subunits or mixtures of β and γ subunits into Swiss 3T3 cells and examined the effect on stress fiber induction. They demonstrated that $G_{\alpha 12}$ and $G_{\alpha 13}$ but not G_{12} nor G_q selectively induced the development of stress fibers and focal adhesions, and that this effect was abolished by C3 exoenzyme. These results indicate that Rho activation is downstream of the G_{12} family heterotrimeric G-proteins. Presumably these G_α proteins transduce a signal to a Rho exchange factor to activate Rho. Nobes *et al.* (*61*) examined this pathway using various enzyme inhibitors. They found that tyrophostin inhibited LPA-induced stress fiber formation without affecting stress fiber formation induced by activated Rho, suggesting that a tyrosine kinase mediates the activation of Rho. This may indicate that G_{12} or G_{13} either activates a tyrosine kinase or inhibits a tyrosine phosphatase. Heterotrimeric G-proteins indeed are linked to tyrosine kinases as shown by the recent finding (*62*) that the tyrosine kinases Lyn and Syk mediate activation of the MAP kinase cascade by G_q and both G_q and G_i, respectively. However, the identity of downstream effectors for G_{12} and G_{13} remains unknown. In contrast to these studies, Coso *et al.* (*63*) showed that $\beta\gamma$ and not α subunits of heterotrimeric G-proteins work to transmit a signal to c-Jun kinase *via* Rac and Ras activation. This appears analogous to the pheromone pathway of *Saccharomyces cerevisiae*, in which Cdc24 is thought to link directly with the $\beta\gamma$ subunits of G-protein (*64*).

As can be imagined from the above discussions, guanine exchange factors for Rho family proteins were not discovered from analyses of upstream signalling in mammalian cells but from studies in yeast. It was known from complementation studies that CDC24 acts upstream of CDC42 in the yeast *S. cerevisiae*. Cerione and collaborators (*65*) noticed that CDC24 contains a region with sequence homology to the *dbl* oncogene product. They examined if the homologous region of Dbl acts on Cdc42 and found that it is capable of stimulating GDP/GTP exchange for this GTPase. It was later shown to have exchange activity also for Rho. Various Dbl-containing molecules were then isolated and some found to have GEF activity for Rho GTPases. They include ect 2 (*66*), ost (*67*), Tiam-1 (*51*), lbc (*68*), and FGD1 (*55*). One reason for this diversity is that most of these factors have limited tissue expression. For example, Dbl is expressed most highly in B-cell lymphoma, Lbc in myeloid leukemia, and Ost in osteosarcoma. Interestingly, all of these molecules show oncogenic activity, which stimulated studies on the oncogenic potential of Rho GTPases as described above. Most of these molecules are activated as oncogenes by deletion of their N-terminal part, which indicates that the N-terminal portion is a regulatory domain of these molecules. However, molecules or specific modifications acting on the N-terminal part to activate GEF activity have yet to be shown. While these Dbl-containing molecules can act on a wide range of Rho family proteins *in vitro* in cell-free systems, they have a limited specificity for one type of protein *in vivo* in the cell. Tiam-1 shows selectivity for Rac, and Lbc for Rho, because upon transfection, they induce membrane ruffling and stress fibers, respectively (*52, 68*). There is another molecule, smg GDS (small GTP-binding protein guanine nucleotide dissociation stimulator), that has no homology to Dbl but shows the GDP-GTP exchange activity *in vitro*. It has some homology to CDC25, shows wider specificity than Dbl homologues and acts on Ki-Ras and Rap in addition to Rho family proteins (*69, 70*). How these exchange proteins are used differently in cells remains unknown.

Another upstream pathway proposed to activate Rho is the small GTPase cascade, but a mechanism by which one GTPase activates another is not known. Peppelenbosch *et al.* (*71*) recently analyzed the mechanism of growth factor-

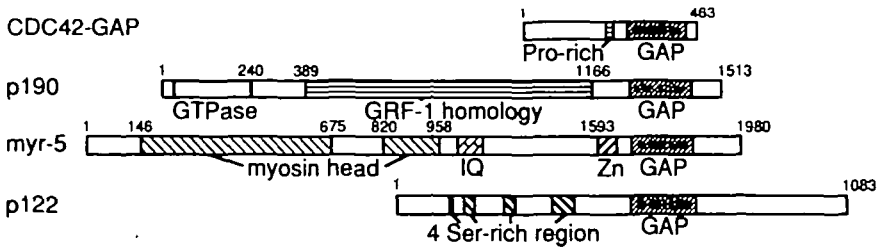

Fig. 3. **Schematic representation of several GTPase activating proteins (GAPs) for Rho family proteins.** Domain structures of Cdc42 GAP [isolated initially as rhoGAP (*79*)], p190 (*83*), myr5 (*82*), and p122 (*81*) are shown.

induced Rac-dependent Rho activation and consequent stress fiber formation. They found in Rat-1 cells that activated Rac induced the release of arachidonic acid which was then converted to leukotrienes. These leukotrienes then induced the activation of Rho and the formation of stress fibers. Because leukotrienes are usually released outside of the cell and act on cell surface receptors, their findings suggest that they work as a paracrine factor between Rac and Rho. Whether it is a major pathway for Rac-induced Rho activation remains an open question. In yeasts, the Ras type of small GTPase can directly bind to an exchange factor for Cdc42; RSR1 acts on CDC24 in *S. cerevisiae* (*72*), and Ras1 acts on Scd2 in *Schizosaccharomyces pombe* (*73*). Interestingly, Ost can bind the GTP-bound form of Rac, indicating that Ost may function as a link between activated Rac and the activation of Rho (*67*).

Another important molecule likely to regulate Rho activation is Rho guanine nucleotide dissociation inhibitor (Rho GDI) (*74*). This is a 23 kDa protein that forms a one to one complex with the Rho family proteins, Rho, Rac, and Cdc42Hs. This protein has two regulatory functions on Rho proteins. One is to inhibit dissociation of guanine nucleotide bound to Rho proteins. Because this protein has more affinity to its GDP-bound form than GTP-Rho, and dissociation of GDP from G-protein is a rate limiting step in the exchange reaction, GDI works as a negative regulator for Rho activation. The other function of Rho GDI is, by making a stable complex, to inhibit Rho from binding to the membrane and to detach GDP-bound Rho from the membrane (*75*). The latter function was shown in the *in vitro* cell-free system, but is consistent with the finding that most of the Rho in the cells is present in the cytoplasm. The negative action of Rho GDI has been demonstrated *in vivo* by microinjection or expression of this molecule in a variety of cultured cell systems, in which Rho-GDI blocked stimulus-induced Rho actions (*26, 31, 76*). Many researchers think that endogenous Rho is present in a complex with Rho GDI in resting cells. If so, the first step in Rho activation may be dissociation of Rho from this complex. To date, the mechanism for triggering this dissociation is not known.

Down regulators. Identification and cloning of Ras GAP suggested the existence of GAPs for other small GTPases, and a distinct GAP activity for Rho was first identified in spleen homogenate (*77*). This GAP was later purified and a partial amino acid sequence of the purified protein showed homology to a part of the breakpoint cluster region (Bcr) gene product (*78*). The finding that a recombinant protein of this region of Bcr showed GAP activity for Rac led to the identification of a variety of molecules containing an homologous sequence (Bcr motif) and displaying GAP activity for Rho family proteins. They show different specificity toward Rho, Rac, and Cdc42 (*79*). For example, the original Rho-GAP is more active on Cdc42 than Rho. The GAPs specific or relatively specific for Rho *in vitro* include p190 Rho-GAP (*80*), p122 Rho-GAP (*81*), and myr5 (*82*). Among them, p190 was shown to inhibit Rho-induced stress fiber formation but not Rac-induced membrane ruffling in cultured cells, indicating that it works selectively on Rho *in vivo*. Interestingly, all of these GAPs have functional domains other than the GAP domain (Fig. 3). For example, p190 has a Ras GAP-binding domain, which when phosphorylated at tyrosine, binds to the N-terminal SH2 domain of p120 Ras GAP (*83*). This association appears to occur *in vivo*, because Ras GAP was recovered in a complex with p190 from lysates of cells after growth factor stimulation. This may indicate that Ras signalling can influence Rho activity in the cell *via* interaction of the respective GAPs. Indeed, it was reported that overexpression of the N-terminal portion of Ras-GAP recruited p190 and downregulated Rho activity to cause a loss of focal adhesions and cell adhesion (*84*). Domains of p122 Rho-GAP and myr5 also indicate connection of Rho to other pathways. p122 was first isolated as PLCδ-binding protein, and was shown to activate this enzyme activity (*81*). While this activation is not influenced by Rho-binding, this molecule may link downregulation of Rho to PLC activation at a specific site in the cell. myr5, a new class of myosin heavy chain isolated by low stringency hybridization screening, contains a myosin head in the N-terminus, an IQ motif in the middle, and Rho-GAP domain and a zinc finger motif near the C-terminus (*82*). This molecule is, therefore, an unconventional myosin which is thought to bind Rho by its tail and to interact with actin through its head. The physiological function of this molecule remains unknown, although it is quite likely that it works in some process of Rho-regulated cytoskeletal reorganization. Thus, there are a variety of Rho-GAPs, which apparently link the downregulation of Rho to other cell functions. It is possible that some GAPs work not only to downregulate activated Rho but also to receive its signal as an effector. However, very little is yet known about the physiological contexts or signalling pathways in which these molecules are employed in the cell.

Downstream signalling. Biochemical pathways involved in the elicitation of Rho actions have been examined by analyzing biochemical events induced by Rho activation and inhibited by C3 exoenzyme treatments and by examining the effects of various enzyme inhibitors on Rho-induced actions. The most frequently used model system is cultured Swiss 3T3 cells stimulated by LPA. Using this system, Kumagai *et al.* (*85*) first found that LPA-induced tyrosine phosphorylation of various proteins, including FAK and paxillin, was inhibited by C3 exoenzyme treatment, indicating that some tyrosine kinase(s) lie downstream of Rho.

This was confirmed in other systems, and by Ridley and Hall (*86*) who found that activated Rho induced tyrosine phosphorylation in focal adhesions. This possible involvement of a tyrosine kinase in Rho-induced focal adhesion assembly and stress fiber formation is consistent with findings by Ridley and Hall (*86*) and by Burridge *et al.* (*87*) that tyrosine kinase inhibitors such as genistein and herbimycin inhibit stimulus-evoked cell adhesion and focal adhesion formation. Kumagai *et al.* (*88*) used a renaturation kinase assay and found that LPA activated several serine/threonine kinases downstream of Rho. They suggested that Rho may directly activate these kinases or drive a kinase cascade. This is consistent with the identification of several serine/threonine protein kinases as putative Rho targets as described below, and may be related to the finding that staurosporine, a broad kinase inhibitor, inhibited LPA- or Rho-induced focal adhesion. Interestingly, this drug, unlike genistein, does not block Rho-induced actin filament assembly, and actin filaments were randomly arranged in the cytoplasm of these cells (*23*). These results indicate that actin filament assembly and focal adhesion formation are regulated by different effectors, both of which may be kinases.

Direct targets for Rho have been looked for by screening molecules selectively binding to, or activated by, GTP-Rho. These studies identified several molecules as putative targets or effectors for Rho. Watanabe *et al.* (*89*) used yeast two hybrid screening combined with ligand overlay assay with [^{35}S]GTPγS-Rho. They identified a serine/threonine protein kinase, PKN, and rhophilin, a molecule containing a Rho-binding sequence homologous to PKN, as putative targets for Rho. Identification of PKN as a putative Rho target was also reported by Amano *et al.* (*90*). Another molecule having a homologous Rho-binding sequence was also isolated and called rhotekin (*91*). Notably, there appeared to be no domain with catalytic activity in rhophilin or rhotekin. These results indicate that there is a family of GTP-Rho binding proteins having the same binding motif but a spectrum of unrelated activities. Another group of GTP-Rho binding proteins was also identified recently. Ishizaki *et al.* (*92*) used ligand overlay assay and purified a GTP-Rho binding protein from platelet cytosol. cDNA cloning for this protein revealed that it is another serine/threonine protein kinase with a molecular weight of 160 kDa. This kinase named p160ROCK (Rho-associated coiled-coil containing kinase) has multiple functional domains, a kinase domain in the N-terminus, followed by a long coiled coil region, and a pleckstrin homology region and a Cys-rich zinc finger in the C-terminus. An isozyme of p160 ROCK was also isolated as a putative Rho target; ROKα by Leung *et al.* (*93*) and Rho kinase by Matsui *et al.* (*94*). These molecules appear to constitute a family, because another coiled-coil containing molecule without a kinase domain, citron, was isolated by a two hybrid system using RhoC as bait (*95*). Structures of these molecules are shown schematically in Fig. 4. The Rho-binding domain of these three molecules appears to localize in the carboxy terminal end of the coiled-coil structure, suggesting the possibility that Rho binding to this region exposes the N-terminal coiled-coil region for complex

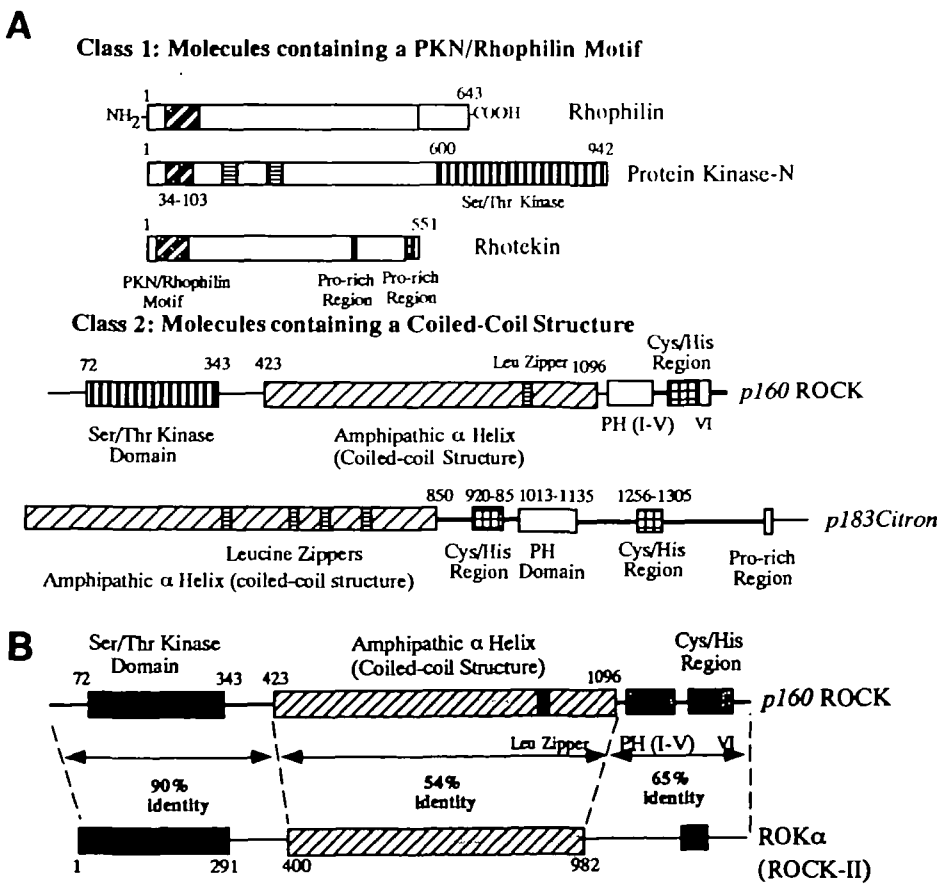

Fig. 4. **A: Schematic representation of two groups of putative Rho target molecules.** Class 1 molecules comprise rhophilin, PKN, and rhotekin. They share about a 70-amino-acid-long homologous Rho binding motif in the N-terminus. Class 2 molecules have a long amphipathic α helix in the middle, that is thought to form a coiled-coil structure. The Rho-binding domain in the class 2 molecules is localized in the C-terminal end of the α helix. **B: Comparison of the structures of p160^{ROCK} and ROKα.** The amino acid sequence homology of p160^{ROCK} and ROKα is compared in three functional domains. ROKα (*93*), an isozyme of p160, is identical to Rho kinase [as reported by Matsui *et al.* (*94*)] and the recently cloned ROCK-II (Nakagawa *et al.*, submitted), although its reported sequence lacks the 86 N-terminal amino acids.

formation and the C-terminal lipid binding region for membrane attachment (*93, 96*). Identification of two serine/threonine kinases as GTP-Rho binding proteins raises a strong possibility that they are the Rho targets as indicated in the above studies. However, direct proof for this has not yet been reported.

In addition to these molecules for which direct binding of GTP-Rho was shown, some molecules were shown to be activated by GTP-Rho in crude membrane or cell lysates. They include PI-4-phosphate 5-kinase, PI 3-kinase, and phospholipase D. Chong *et al.* (*97*) demonstrated that GTPγS-loaded Rho added to lysates of C3H fibroblasts increased the activity of PIP-5-kinase. This is particularly interesting since the product formed by this reaction, PIP2, binds to a variety of actin binding proteins and is thought to regulate actin filament assembly by uncapping barbed ends of actin filaments and releasing actin monomers (*98*). Indeed, Hartwig *et al.* (*99*), using permeabilized platelets, found that activated Rac and not Rho mediated thrombin-induced phosphoinositide formation, which then facilitated uncapping of actin barbed ends and triggered actin filament elongation. Consistently, Rac was shown to bind to PIP-5-kinase both *in vitro* and *in vivo* (*100*). The inability of Rho to activate and to bind to PIP-5-kinase in the above studies may indicate that Rho interacts with a different isoform of the enzyme or that the effect of Rho is indirect. A similar finding was also reported for the activation of PI-3-kinase by GTP-Rho. Zhang *et al.* (*101*) found that GTP-Rho added to platelet lysates activated PI-3-kinase. However, again, no direct binding of Rho to this lipid kinase was reported, and the effect may be indirect. Nobes *et al.* (*61*) examined the role of PI-3-kinase activation in Rho actions by using wortmannin, a relatively specific inhibitor of this enzyme, and reported that this drug had no effect on Rho-induced formation of stress fibers and focal adhesions, although it inhibited PDGF-induced membrane ruffling.

Reconstitution experiments revealed that Rho also activates PLD in several systems. It was known for some time that PLD activity was enhanced by the addition of GTP and/or cytosolic factor. Examination of these effects revealed the involvement of two small GTPases in this activation, *i.e.* ARF and Rho. Involvement of Rho was verified by inhibition of the activation by Rho-GDI and/or C3 exoenzyme and restoration by adding GTP-Rho in the systems (*102, 103*). It now appears that there are PLD species sensitive to ARF or Rho alone, or to ARF and Rho. An isoform of mammalian PLD was recently cloned. This enzyme was reported to be activated by ARF and Rho as well (*104*). PLD is a signal transducer working in the transmembrane signalling system to generate second messenger molecules. Involvement of Rho in 5HT1c receptor-mediated transmembrane signalling was demonstrated previously (*105*). PLD may be involved in this process. Whether it is also involved in other Rho actions remains to be examined.

Rho signalling in yeast. There are five *RHO* genes in the yeast *S. cerevisiae*, *RHO1* to *4* and *CDC42*. Among them, *RHO1* is a yeast homologue of mammalian *rho* genes (*106, 107*). *RHO1* is an essential gene, as its disruption causes death. Involvement of *RHO1* in the budding process was suggested by the findings that a diploid strain containing an activated allele of *RHO1* did not sporulate (*106*) and that a strain with a temperature-sensitive mutant of *RHO1* showed growth arrest with the phenotype of a tiny bud (*108*). Expression of an epitope-tagged Rho1p and subsequent immunocytochemistry showed Rho1p localization on the tip of a growing bud and at the site of cytokinesis (*108*). Although Rho1p co-localizes with actin microfilaments at these sites, there has been no conclusive evidence that Rho1p is involved in actin cytoskeleton organization as found in mammalian cells. Qadota *et al.* (*109*) substituted *RHO1* in *S. cerevisiae* with human *rhoA*. This strain showed conditional lethality, being unable to grow at 37°C due to osmotic fragility at this temperature. Osmotic fragility is usually caused by some defect in cell walls, indicating that Rho1p is involved in cell wall biosynthesis. Nonaka *et al.* (*110*), taking the same approach, screened for dominant mutations suppressing this phenotype and isolated a mutant of *PKC1*, an yeast protein kinase C homologue, as a suppressor. They further showed that Pkc1p directly interacted with GTP-Rho1p in a two hybrid system. *PKC1* directs the activation of an yeast MAP kinase cascade *MKK1* and *MPK1*, and dominant active mutants of these kinases also suppress the *rhoA* phenotype, indicating that Rho1p is indeed located upstream of this kinase pathway. This is consistent with the osmotic fragility seen in the *rho*A mutant, because *pkc1* null mutants show also osmotic fragility and are rescued in media of high osmolarity (*111*) and a genetic interaction has been shown between the *PKC1* pathway and some cell wall synthesizing enzymes (*112*). *RHO1* is not just indirectly involved in yeast cell wall synthesis through the PKC1 pathway. It has been reported recently that Rho1p directly regulates the activity of a cell wall synthesizing enzyme (*113, 114*). One of the main structural components of the yeast cell wall is 1,3-β-linked glucan polymers, which are synthesized by 1,3-glucan synthase encoded by Fsk1 and 2. Because it is known that this glucan synthase is activated by GTPγS, Drgonová *et al.* (*113*) and Qadota *et al.* (*114*) examined the role of Rho1p in this activation. Both of them found the decreased activity of glucan synthases from the *rho1* mutants which were insensitive to GTP. This defect was corrected by adding the wild type Rho1p to the incubation. Qadota *et al.* (*114*) further showed that Rho1p made a complex with the Fsk1 subunit of the enzyme, co-localizing at the bud tip where the cell remodels its wall. Thus, *RHO1* in yeast exerts two distinct actions in the yeast cell wall synthesis; activation of the PKC1 pathway to induce glucan synthetases and association with Fsk1p to activate the enzyme directly. It is quite likely that Rho1p drives other pathway(s) in yeast, because *rho1* null mutants are not rescued in media of high osmolarity (*108*). Whether such a pathway links Rho to actin assembly in yeast remains to be elucidated.

Perspectives: A model of the mechanism of action of Rho

On the basis of the various findings summarized above, I propose a model describing the mechanism of action of Rho (Fig. 5). It is now evident that Rho exerts a variety of actions in the cell and it is quite likely that Rho performs these actions by interacting with many molecules. We hypothesize that Rho actions on nuclear signalling and changes to the actin cytoskeleton are separate and independent. The former pathway is likely to be mediated by a kinase cascade similar to the PKC1 pathway in *S. cer-*

evisiae. As described, mammalian Rho can activate two serine/threonine kinases, PKN and p160^{ROCK} and related molecules. If the Rho-driven kinase cascades has been conserved in evolution, PKN is a strong candidate as a Rho effector in this pathway, because it has good homology to PKC in its catalytic domain. Then, how does Rho direct organization of actin cytoskeleton? Rho appears to induce several different types of actin cytoskeleton. Assembly of stress fibers and focal adhesions, formation of the contractile ring, and neurite retraction look quite different. However, three elemental functions, *i.e.* site determination, actin polymerization and contractile response, appear to be required in organization of any of these. Based on the above discussions, we speculate that these functions are exerted by Rho. For example, in the formation of the contractile ring, we speculate that Rho determines where the actin cytoskeleton is constructed, induces actin polymerization there and generates the force for contraction. Similarly, Rho performs the first two functions in the assembly of focal adhesions and stress fibers (Fig. 5). The site determined for this process, however, is different from that chosen for contractile ring formation. Therefore, it appears that, in cell adhesion and cytokinesis, Rho drives the same machinery for actin polymerization but at different places in the cell. The hypothesis that the actions of Rho on actin polymerization and site selection are linked but independent is supported by their different sensitivity to staurosporine and by their different ECM protein requirement as discussed above. The model in Fig. 5 hypothesizes that Rho action on actin polymerization is mediated by PIP-5-kinase which is recruited by Rho to specific sites in the cell. PIP-5-kinase generates PIP2 at these sites, which binds to actin binding proteins to release actin monomers and to uncap barbed ends of F-actin to promote actin polymerization. A WASP-like proline-rich molecule may also be involved in this process, because it may recruit profilin at the site of Rho action. Rho-induced actin polymerization may be terminated by p122 Rho-GAP which not only downregulates Rho by accerelating GTP hydrolysis but also attenuates actin polymerization through hydrolysis of PIP2 by its associated PLCδ. How then is the site selection performed? In the case of focal adhesion formation, this process is reported to be sensitive to staurosporine, suggesting a role for a serine/threonine kinase. p160^{ROCK} or related kinases may work in this process, because myotonic dystrophy protein kinase, a kinase homologous to p160, is enriched in places such as dense plaques of smooth muscles and intercalated discs of cardiac muscles where many focal adhesion proteins also accumulate (*115*). p160 may also have a role in site determination, because it may bind to membrane PIP2 *via* its PH domain and may form a complex with other proteins *via* its amphipathic α-helix region. The model hypothesizes that once phosphorylation occurs at the selected sites, p160 induces the activation of integrin molecules there. This is followed by the ligation of integrins with ECM proteins, triggering the assembly of multi-protein complexes (*20, 116*). Incorporation and activation of a tyrosine kinase or kinases in this complex would stabilize the structure of the complex and actin fibers. As discussed above, different sites are chosen for focal adhesion formation and contractile ring formation. This may be due to the difference in the target molecule, the docking molecule for this target, or both. As described previously, the involvement of a serine/threonine kinase has also been suggested in the Rho-induced increase in calcium sensitivity. p160 or related kinases may participate in this process. Kaibuchi and collaborators (*94*) made a preliminarily report that their Rho kinase equivallent to ROKα (*93*) or ROCK-II (*117*) phosphorylate the p130 myosin binding subunit of MLC phosphatase, thereby inactivating its phosphatase activity to enhance interaction of the actomyosin system.

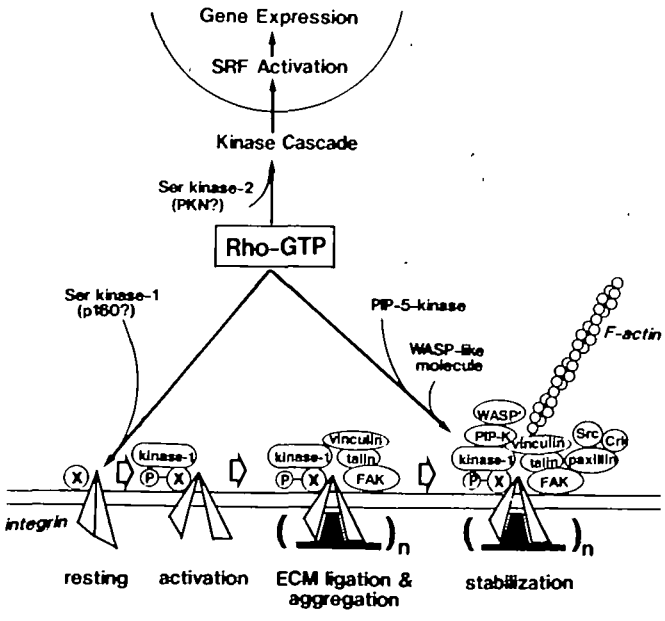

Fig. 5. **A model of the mechanism of action of Rho.** In this model, Rho acts on two serine/threonine kinases. One of them (kinase-2) activates a kinase cascade, which transmits a signal to nuclei for SRF activation. The other (kinase-1) is recruited by GTP-Rho to the membrane, in which it forms a complex with a anchoring or scaffold protein and phosphorylates this protein or a different membrane substrate protein (X). The phosphorylated protein interacts with the cytoplasmic portion of integrin and this interaction activates integrin molecules. Activated integrins then bind to extracellular matrix proteins, and this process causes aggregation of integrins, triggering the sequential assembly of focal adhesion complexes. GTP-Rho recruits PIP-5-kinase and, possibly, a WASP-like proline-rich protein (WASP') at the site of complex formation. These molecules release actin monomers, uncap actin filaments by the action of PIP2, and trigger actin polymerization to form stress fibers. Tyrosine kinases such as FAK, Src, or possibly others come into this complex, and phosphorylate various proteins to stabilize these structures and make signalling complexes including SOS and Ras.

Conclusion

Significant progress has been made in Rho research in recent years. Modes of Rho actions have been characterized in more details and several putative target molecules have been identified. Based on these progresses, a model for biochemical mechanism of Rho actions such as that shown in Fig. 5 has been constructed. However, precise roles of these putative targets have to be defined by constructing dominant positive and negative forms of each molecule and by use of specific inhibitors and antibodies. In addition, these are much to do to identify the pathway for Rho activation following receptor-G-protein interactions. Much efforts will be paid also to examine functions of Rho regulators and targets in the body. These studies will

certainly facilitate our understanding not only on the fundamental mechanisms of cell adhesion and motility but also on the physiological significance of Rho-mediated processes in the whole body.

The author thanks Dr. Pascal Madaule for his critical reading of this manuscript and Miss Keiko Okuyama for secretarial assistance.

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