Models of the Cooperative Mechanism for Rho Effector Recognition

IMPLICATIONS FOR RhoA-MEDIATED EFFECOR ACTIVATION*

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Activated GTPases of the Rho family regulate a spectrum of functionally diverse downstream effectors, initiating a network of signal transduction pathways by interaction and activation of effector proteins. Although effectors are defined as proteins that selectively bind the GTP-bound state of the small GTPases, there have also been several indications for a nucleotide-independent binding mode. By characterizing the molecular mechanism of RhoA interaction with its effectors, we have determined the equilibrium dissociation constants of several Rho-binding domains of three different effector proteins (Rhotekin, ROCKI/ROK1/PRK1, where ROK is Rho-associated kinase) for both RhoA-GDP and RhoA-GTP using fluorescence spectroscopy. In addition, we have identified two novel Rho-interacting domains in ROCKI, which bind RhoA with high affinity but not Cdc42 or Rac1. Our results, together with recent structural data, support the notion of multiple effector-binding sites in RhoA and strongly indicate a cooperative binding mechanism for PRK1 and ROCKI that may be the molecular basis of Rho-mediated effector activation.

The Rho family GTPases act as tightly regulated molecular switches governing critical cellular functions (1–4). Their activity is controlled by two biochemical reactions, the GDP/GTP exchange and the GTP hydrolysis. The formation of the active GTP-bound state of the GTPase is accompanied by conformational changes of two regions (called switch I and II) that provide a platform for the selective interaction with effector proteins (5, 6) and thereby initiate downstream signaling. The fact that a variety of potential effectors have been identified for a single GTPase renders the GTPase-effector interaction the most fundamental and pivotal event in signal transduction (3, 7–11).

Several effector molecules for RhoA have been identified so far that are either scaffold proteins such as rhophilin, Rhotekin, kinectin, and diaphanous or serine/threonine protein kinases such as protein kinase C-related kinase (PRK1, also called protein kinase No or PKNα), citron kinase, and Rho-associated coiled-coil kinase (ROCK) (9, 11). RhoA binds to RhoG in a GTP-dependent manner (5), indicating that effector binding involves the switch regions of the GTPases. This is confirmed by the recent crystal structure of the complex between active RhoA and the Rho-binding domain (RBD) of ROCKI. ROCKI-RBD forms a parallel α-helical coiled-coil dimer and binds exclusively to the switch I and switch II regions of RhoA (6).

In contrast to Rhotekin (28) and the ROCK proteins (29, 30), which contain only one Rho-binding domain, two leucine zipper motifs (homology regions (HR) 1a and 1b) have been found to be responsible for the interaction of PRK1 with Rho (see Fig. 1A) (31, 32). The structure of PRK1-HR1a in complex with RhoA revealed that the HR1a domain forms an antiparallel coiled-coil (ACC) and is able to bind to two distinct surface sites of RhoA (33), one within and the other apart from the switch regions. An ACC-like structure has also been predicted for other HR1-containing proteins such as rhophilin and Rhotekin (33) and has been recently confirmed for PRK1-HR1b (34).

The most common mechanism of effector activation by Rho GTPases appears to be the disruption of intramolecular autoinhibitory interactions to expose functional domains within the effector protein (9, 21, 35). Although considerable progress has been made in elucidating physiological processes involving Rho effector signaling pathways, the molecular basis of RhoA interaction with its effectors has not been thoroughly characterized yet.

In this study, we present binding constants for the interaction of individual effector RBDs with the active and inactive forms of RhoA determined by fluorescence spectroscopy. We also present data for two novel Rho-interacting domains of ROCKI, which tightly interact with RhoA but not with Rac1.
and Cdc42. These data, together with the available structural data on PRK1 and ROCKI, make us propose a cooperative activation model for Rho effector kinases.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Human RhoA (amino acids 1-181; accession number L52080; human PRK-HR1a (1-106) (Q16512); HR1b (122-199); human ROCK-RBD-HR1a (947-1015) (U34195); ROCK-RID-HR1 (842-931); ROCK-HR1-H18528 to 1-89) (U54638) were cloned in pEXEMT1 and pQE32 via BamHI/XhoI sites, respectively.

**Proteins**—RhoA, PRK-HR1a, PRK-HR1b, ROCK-RBD, ROCK-RID, ROCK-HR1, and Rocknetin-HR1 were produced as glutathione S-transferase (GST) or His-tagged fusion proteins in *Escherichia coli* as described (36–38, 40, 41). His-tagged proteins were purified by nickel-nitrotriacetic acid-agarose affinity chromatography. After thrombin cleavage and concentration with centrifugal concentrators (Vivaspin 5, 10, or 30-kDa molecular weight cut-off, Viva Science) the proteins were purified by size exclusion chromatography (Superdex 75, Amersham Biosciences) at a final purity of at least 95%. Rac and PAK1(57-241) were purified as described (37, 38). His-tagged proteins were purified by nickel-nitrotriacetic acid-agarose affinity chromatography (Qiagen) with subsequent concentration and size exclusion chromatography to a similar purity. The protein quality and the nucleotide binding capacity of RhoA were analyzed by SDS-PAGE and reversed-phase HPLC using a C-18 column (ODS-Hypersil, 5 μm, Biscoff) as described (36). Proteins were snap-frozen in liquid nitrogen and stored at 80 °C in 30 mM Tris/HCl, pH 7.5, 3 mM dithioerythritol, and 5 mM MgCl2. Protein masses were confirmed by electrospray ionization mass spectrometry (Finnigan). The obtained molecular masses for the respective proteins corresponded to that calculated by the Genetics Computer Group program teptidesort (Madison, WI).

**Nucleotide Exchange**—Nucleotide-free Rho proteins were prepared using enzymatic activity of alkaline phosphatase (Roche Applied Science) and phosphodiesterase (Sigma) at 4 °C as described previously for Ras (39). mantGDP- and mantGpp(NH)p-bound Rho proteins were prepared by mixing the nucleotide-free form of the respective proteins and the fluorescent nucleotides in a molar ratio of 1:1.5 and using prepacked gel filtration columns (NAP5, Amersham Biosciences) to remove unbound nucleotides. The concentrations of the nucleotide-bound proteins were determined by HPLC as described (36).

**Guanine Nucleotide Dissociation Inhibition Measurements**—The inhibition of the mantGDP or mantGpp(NH)p release from RhoA (0.2 μM) by increasing amounts of the respective effector domains (0.01–300 μM) in the presence of unlabeled nucleotide (40 μM) was monitored on a LS50B PerkinElmer Life Sciences spectrophotometer as described previously for Ras-Raf and Rac-PAK interactions (36–38, 40, 41). All measurements were carried out at room temperature with mantGDP or NaHPO4, pH 7.5, and 5 mM dithiothreitol at 25 °C. The observed rate constants (kobs) were single exponentially fitted using the program Grafit (Erithacus software). The dependence of the observed rate constants on the effector concentration was fitted to the dissociation constant (Kd) as described (36, 41).

**RESULTS**

**Quantitative Measurements of Rho-Effector Interaction**—The interaction between Rho effectors and RhoA has been primarily investigated in vitro either with solid phase methods such as radioactive ligand overlay or pull-down assays (14, 28, 30, 32, 42-44) or with yeast two-hybrid systems (30, 43, 45, 46). Although these techniques have obviously been very valuable in identifying the binding partners in the first place, they do not provide any information about the affinity of the binding partners or the time-resolved detection and quantification of the Rho-effector interactions. However, no fluorescence change could be observed upon mixing RhoA with the respective effector RBDs using various fluorescently labeled nucleotides or tryptophan fluorescence of the GTPase. The crystal structures of the RhoA-GTP-γS/PRK-HR1a (33) and RhoA-Gpp(NH)p-ROCK-RBD complexes (6) revealed that the binding of the RBDs does not influence the environment of the fluorescence reporter groups, and therefore, no change in fluorescence occurred. Thus, we took advantage of the ability of effector domains (Fig. 1A) to inhibit the guanine nucleotide dissociation from the GTPase upon binding (so-called guanine nucleotide dissociation inhibition effect). This method, which provides a large fluorescence change upon the release of mant-nucleotide, has been successfully used for quantitative analysis of the effector interactions for Ras (40, 41, 47, 48), Cdc42 (49), and Rac proteins (37, 38). An incremental inhibition of the mantGpp(NH)p release from RhoA was observed when the measurements were carried out in the presence of a 200-fold excess of unlabelled Gpp(NH)p and increasing concentrations of effector RBDs. As shown in Fig. 1B, the nucleotide dissociation rate decreases with increasing ROCK-RBD concentrations. With the Rocknetin-HR1 and HR1a and HR1b of PRK1, we observed different degrees of nucleotide dissociation inhibition (data not shown).

The dependency of the observed rate constant (kobs) on the concentration of the respective effector domains (Fig. 2, A–D), evaluated according to Herrmann et al. (40, 41), allowed the determination of equilibrium dissociation constants (Kd) for the RhoA-mantGpp(NH)p interaction with ROCKI-RBD, Rocknetin-HR1, PRK-HR1a, and PRK-HR1b, respectively. As summarized in Table I, there is a remarkable difference in the binding of Rocknetin-HR1 and PRK-HR1b to RhoA, which represent with 0.009 and 1.8 μM the highest and the lowest binding affinity. In contrast, ROCKI-RBD and PRK-HR1a bind with a strikingly similar affinity to the active state of RhoA (Table I).

Unlike Rocknetin (28), the ROCK proteins (29, 30) and PRK-HR1a, the PRK-HR1b domain has been reported to bind RhoA in a nucleotide-independent manner (32). Moreover, the HR1a domain of PRK1 has been reported to contact RhoA at a region that does not undergo any conformational change upon GDP/GTP exchange or GTP hydrolysis (33). To assess the GTP dependence of the RBD binding, we also determined the binding affinity of the respective effector domains for inactive RhoA using the effector-mediated inhibition effect on the mantGDP dissociation (Fig. 2, E–H). A comparison of the measured Kd values, which are summarized in Table I, indicates that Rocknetin exhibits the highest selectivity for the RhoA-GTP. A comparable GTP dependence of 2 orders of magnitude was reported previously for the Rac interaction with its effectors Raf kinase and RalGDS (40, 41). The strong preference of Rocknetin for the GTP-bound RhoA also justifies the usage of the Rocknetin-HR1 in GST pull down experiments to fish activated RhoA from cell lysates (28, 50). However, it is important to note that the affinity of GST-Rocknetin-HR1 for RhoA-mantGpp(NH)p is reduced about 16-fold (0.15 μM; data not shown) due to steric hindrance of the 26-kDa GST anchor compared with the relatively small RBD (10 kDa).

In contrast to Rocknetin, the GTP-dependent binding to RhoA is less pronounced for ROCK-RBD (10-fold) and PRK-HR1a (4-fold) (Fig. 2 and Table I). The determined affinities for ROCK and PRK1 are comparable with what has been reported previously for the interaction of Cdc42 and the Rac isoforms with their effectors analyzed under equilibrium conditions using scintillation proximity assay, isotermal microcalorimetry, or fluorescence microscopy (37, 38, 51–53). PRK-HR1b exhibits its preference for the GTP-bound form of RhoA, but the respective Kd values are only in the low micromolar range (Table I). Our data are in good agreement with the Kd values for the interaction of the HR1 domains of PRK1 with the constitutive active RhoAQ61L-GTP, recently reported by Owen et al. (34).

**A Second Rho-interacting Domain (RID) Identified in ROCK**—The contact sites outside the switch regions have been implicated not only to determine the specificity of the GTPase-effector interaction but also to contribute to effector activation (5, 6, 54). There have been several reasons why we have been
searching within all ROCK sequences for additional conserved regions that may contribute to Rho-binding. (i) ROCK proteins control various physiological and pathophysiological functions (21, 55), emphasizing the crucial regulatory role of Rho in binding and activating these kinases. (ii) ROCK has been reported to bind two different regions of RhoA (56), which are both partially occupied by the ROCK-RBD as shown by the recent crystal structure (6). (iii) ROCK-RBD binds RhoA/GTP with a moderate affinity that is comparable with that of the HR1a domain of PRK1 (Table I), which in turn provides with HR1b a second Rho-binding domain most likely necessary to accomplish a tight interaction. (iv) An N-terminally elongated fragment of the ROCK-RBD (amino acids 920–1024) has been shown to display better Rho binding properties than the ROCK-RBD itself (30). (v) As GTP dependence of ROCKI-RBD binding to RhoA is not as pronounced as expected for an effector interaction, it seems likely that GTP-dependent binding is achieved by more than one binding domain.

Thus, we addressed possible RhoA binding activities of additional protein fragments upstream of the ROCKI-RBD. We found a domain encompassing amino acids 842–931 adjacent to the ROCKI-RBD (called RID; Fig. 1A), which is highly conserved among all ROCK proteins (>80% homology). As shown in Fig. 3A, the ROCKII-like proteins show deviations in the middle part of the fragment caused by amino acid insertion and deletion, whereas the N-terminal and C-terminal parts of the RIDs are highly homologous in all ROCK isoforms. Moreover, this domain does not exist in ROCK proteins alone but seems to be also conserved in other effectors such as kinectin and citron (Fig. 3A). ROCKI-RID is indeed able to bind RhoA, thereby inhibiting mantGpp(NH)p dissociation, but does not interact with Cdc42 and Rac proteins (data not shown). The characterization of the interaction of RhoA with RID showed a GTP-dependent binding with $K_d$ values of 0.26 $\mu$M for RhoA/mantGpp(NH)p and 1.1 $\mu$M for RhoA/mantGDP, respectively (Fig. 3, A and B, and Table I). Our data indicate that Rho binding of ROCK also involves two adjacent binding domains (RID-RBD) as shown for PRK1 (HR1a-HR1b), but how ROCK utilizes both RBD and RID to interact with the GTP-bound RhoA remains to be elucidated.

**Fig. 1.** Fluorescence-based measurement of the RhoA interaction with various effector domains. A, schematic overview of the ROCKI, PRK1, Rhotekin, and HR1 domain architecture. The effector domains (dark boxes) that have been used in this study are indicated by the black bars and the N-/C-terminal amino acid numbers. The text amphipathic $\alpha$-helix indicates a common domain of predicted coiled-coils, and Kinase indicates the catalytic domain. C2, Ca$^{2+}$-dependent lipid-binding domain; CRD, cysteine-rich domains; $P$, proline-rich region; $PH$, pleckstrin homology domain. B, guanine nucleotide dissociation inhibition assay. Inhibition of mantGpp(NH)p dissociation from RhoA was measured in the absence ($\odot$) and in the presence of 1.25 ($\bullet$), 2.5 ($\square$), 3.75 ($\oplus$), and 5 $\mu$M ($\triangle$) ROCK-RBD after the addition of 40 $\mu$M unlabelled Gpp(NH)p. RhoA/mantGpp(NH)p concentration was 0.2 $\mu$M. The lines through the data points were obtained by single exponential fitting and represent the determined observed dissociation rate constants ($k_{obs}$). rel, relative.
To examine the binding capability and affinity of the ROCKI-HR1 domain, we successfully purified a ROCK fragment containing the HR1 domain (amino acids 420–550) from E. coli. As shown in Fig. 4, B and C, ROCK-HR1 binds to both the mantGpp(NH)p-bound and the mantGDP-bound form of RhoA with comparable affinity (Table I). The fact that the ROCKI-HR1 domain shares a certain degree of homology with both HR1 domains of PRK1 and binds RhoA in a nucleotide-independent manner strongly indicates that its interaction with RhoA take place outside the switch regions.

**DISCUSSION**

The interaction of RhoA with a large and functionally diverse number of effectors is the basis for signal transduction in distinct cellular pathways. In addition to the competition of multiple effectors for GTPase binding, the utilization of multiple contact sites adds another level of complexity toward comprehending the molecular mechanisms behind RhoA-mediated effector activation. To investigate the key principles of complex formation between RhoA and its effectors, we have determined equilibrium dissociation constants for the nucleotide-dependent interaction between RhoA and six different effector domains of Rhotekin, PRK1, and ROCKI. Our data, summarized in Table I, show that all domains except for the ROCK-HR1 domain bind with a higher preference to the GTP-bound state of RhoA. However, only Rhotekin-HR1, which binds RhoA-GTP with the highest measured affinity (9 nM), exhibits a GTP dependence expected for an effector interaction (110-fold; Fig. 2, B and F). Interestingly, ROCKI-RBD and PRK1-HR1a, which neither share any sequence homology nor adopt similar structural folds (except for exhibiting a coiled-coil structure), revealed comparable binding constants. The two ROCK domains HR1 and RID, described first time in this study, bind RhoA with reasonably tight affinities, which is, in the case of the ROCK-HR1 domain, independent of the bound nucleotide.

The HR1-like domains seem to play an important role in the effector interaction with Rho GTPases as they are found in PRK proteins (59), rhophilin (43), Rhotekin (28), and now in ROCK and apparently in citron and kinectin (Fig. 4A). The HR1 domain was first described as a three times repeat (HR1a, b, and c) of the N-terminal non-catalytic part of PRK1 (59), but only the first two of these repeats have been shown to bind RhoA (31, 32, 34). The HR1a and HR1b structures of PRK1 have been determined by x-ray crystallography and NMR (33, 34), and they form an ACC fold that has also been suggested for other Rho-binding HR1 domains (33). The crystal structure of PRK1-HR1a in complex with RhoA<sub>GTP</sub> (33) interestingly reveals two alternative contact sites on RhoA (called contact sites I and II). Contact site II nicely overlaps with the binding site of ROCKI-RBD comprising the switch regions (6), whereas contact site I is formed by the α1, β2/β3, and α5 regions of RhoA (Fig. 5A), which do not undergo any conformational change on
HR1a and HR1b binding to RhoA characterized in this study, particularly in the I and II regions of RhoA (contact site II, Fig. 4) series of hydrophobic and electrostatic contacts with the switch compartment, where it functions as a scaffold protein (Fig. 5 switch regions of RhoA is sufficient to recruit it to the cellular that a tight GTP-dependent binding of Rhotekin-HR1 to the tional Rho-binding domain has been reported so far, we propose Rhotekin, neither an internal inhibitory domain nor an addi- tivity binding of Rhotekin-HR1. Irrespective of the fact that for a stronger interaction and may be responsible for the high affin- ity binding of Rhotekin-HR1. Asn-58, Lys-49, Lys-51, Leu-52, and particularly Asn-58 of exhibits a few striking deviations in comparison with the Rho- tectin-HR1. Lys-48, Arg-68, Val-73 in HR1a, which make crucial to the nucleotide-dependent conformational change of RhoA. Interestingly, the HR1a region of PRK1, which makes a series of hydrophobic and electrostatic contacts with the switch I and II regions of RhoA (contact site II, Fig. 4A, gray square), exhibits a few striking deviations in comparison with the Rho- tectin-HR1. Lys-49, Lys-51, Leu-52, and particularly Asn-58 of PRK1 are replaced by His-34, Arg-37, Met-38, and Lys-44 in Rhotekin. Asn-58 of PRK1-HR1a contacts Glu-40 of switch I in RhoA, a residue critical for the GTP-dependent interaction of PRK1-HR1a and possibly Rhotekin-HR1 with RhoA. Mutational studies have shown that substitution of Glu-40 disrupts PRK1 binding to RhoA (60). As Rhotekin contains a lysine (Lys-44) instead of an asparagine in PRK1-HR1a (Asn-58), this may cause a stronger interaction and may be responsible for the high affinity binding of Rhotekin-HR1. Irrespective of the fact that for Rhotekin, neither an internal inhibitory domain nor an additional Rho-binding domain has been reported so far, we propose that a tight GTP-dependent binding of Rhotekin-HR1 to the switch regions of RhoA is sufficient to recruit it to the cellular compartment, where it functions as a scaffold protein (Fig. 5B).

In contrast to Rhotekin that contains one RBD, PRK proteins provide three HR1 domains that reveal different binding char- acteristics. Unlike HR1c, which does not interact with RhoA at all (32), HR1a binds with a significantly higher affinity to RhoA-GTP than HR1b (Fig. 2, C and D, and Table I). Our data are consistent with the binding constants of 0.28 and >1 μM for HR1a and HR1b binding to RhoA-GTP determined by scintillation proximity assay (34). In addition, it has been shown that a PRK1 fragment encompassing HR1a-HR1b domains reveals a stronger binding of PRK1 to RhoA than the respective isolated domains (32). This group has also shown that HR1b interacts with RhoA in a nucleotide-independent manner using a ligand-overlay assay (32). This method is obviously not sen- sitive enough to discriminate against a 7-fold different Kd val- ues (Table I). Taken together, it seems that two PRK1-HR1 domains may cooperatively approach the two binding sites of RhoA (Fig. 5, A and C). Thus, we propose that the HR1a domain first interacts with RhoA-GTP by recognizing and binding to its switch regions. In a second step, the HR1b domain binds to the contact site I (Fig. 5C), probably causing the release of autoinhibitory contacts and consequently activating these protein kinases.

A sequence comparison of the two PRK-HR1 domains shows tremendous differences at several positions especially at positions Lys-48, Arg-68, Val-73 in HR1a, which make crucial contacts to the switch II and α2 helix of RhoA (Fig. 4A, gray squares). Lys-48 and Arg-68 make salt bridges with Asp-65 and Asp-76, respectively, whereas Val-73 contacts Leu-72 at the hydrophobic patch. Since Lys-48 and Val-73 of the HR1a domain are replaced by isoleucine and lysine in HR1b, and Arg-68 does not exist in HR1b (33), a similar binding mode of HR1b to the switch regions can be excluded.

Interestingly, the amino acids that participate in the contact site I interface (Fig. 4A, black circles) are not well conserved among the HR1 domains of PRK proteins. It has been shown that an E55A/N56A (Glu-57 and Asn-58 in PRK1-HR1a) double mutant of human rhophilin-2 abolishes binding to RhoA (14), whereas G58A (Gly-55 in PRK1-HR1a) mutant of Drosophila Pkn (a PRK1 ortholog) had no effect on Rho1 but on Rac binding (61). In contact site I, PRK1-HR1a, only Asn-58 directly engages Arg-168 of RhoA with its carboxamido-oxygen. However, these residues (Gly-55, Glu-57, Asn-57 in HR1b) may be involved in the same interaction as they are also conserved in the HR1b domain. Considering contact site I, the most conspicuous amino acid deviations between both HR1 domains of
PRK1, concerning RhoA-binding residues, are 4 arginines at position 47, 60, 61, and 78 in HR1a, which are replaced by alanine, isoleucine, glutamine, and alanine in HR1b (Fig. 4A, black circles). In HR1a, the first 2 participate in electrostatic interactions with Gln-52 and Glu-169 of RhoA, whereas the other 2 arginines are supposed to stabilize the complex by coordinating water molecules (33). The loss of these 2 interactions could result in the 10-fold decrease in affinity observed for HR1b (Fig. 2C and Table I), although it is not clear whether HR1b binds in the same way seen for HR1a in the complex structure (33).

In the case of the ROCK protein, a new situation of a three-domain ROCK interaction with RhoA has emerged from the identification and analysis of the two additional ROCK domains, RID and HR1 (Figs. 3A and 4A), beside the well known ROCK-RBD. The existence of three binding domains supports the presumption of multiple binding sites on RhoA. To gain insight into the ROCK-RBD binding mode, we recently solved the crystal structure of RhoA\textsuperscript{H18528}/Gpp(NH)p in complex with ROCKI-RBD (6), which shares no sequence homology with any other Rho-specific effector protein. The ROCKI-RBD dimer employs a minimal Rho-interacting motif of a 13-residue left-

**Fig. 4. Characterization of a novel HR1 domain of ROCKI.** A, sequence alignment of several HR1 domains (program Genedoc (74)). Identical amino acids are boxed in gray. The numbers at the right side correspond to the very C-terminal amino acids of the proteins. Black circles and gray squares indicate the interacting residues of PRK1-HR1a with RhoA at contact site I and II, respectively, according to the Rho-HR1a structure (33). α2 and α3 helices of the PRK1-HR1a ACC finger (33) are shown as gray boxes. Cf, Canis familiaris; Hs, H. sapiens; rn, R. norvegicus. B and C, plot of the $k_{cat}$ values of RhoA\textsuperscript{mantGpp(NH)p} (B) and RhoA\textsuperscript{mantGDP} (C) against the ROCKI-HR1 concentration. The $K_d$ values for the Rho-RID interactions obtained as in Fig. 3 are listed in Table I.

**Fig. 5. Proposed model RhoA-effector binding mechanisms.** A, effector binding sites of RhoA. A surface representation of the RhoA\textsuperscript{Gpp(NH)p} structure illustrates the switch I and II regions as the ROCK-RBD-binding site (6) superposed with the PRK-HR1a contact site II (33) in orange, the PRK-HR1a contact site I (33) in dark red, and the asparagines 87 and 90 in the loop 6 in yellow. B–D, proposed models for Rhotekin binding (B), PRK binding (C), and ROCK binding (D) to RhoA.
handed parallel coiled-coil structure, which binds exclusively to the switch I and switch II regions of two RhoA molecules (Fig. 5, A and D) (6). The switch regions that are essential determinants of the GTP-dependent interactions of RhoA with its effectors (62) are the reason for a 10-fold higher affinity of the ROCK-RBD for RhoA-Gpp(NH)p than for RhoA-GDP (Fig. 2, A and E, and Table I). In agreement with the structural data and previous mutational studies on RhoA and the ROCK proteins (32, 60, 63, 64), our data confirm the switch regions of RhoA as the prime binding site of the ROCK-RBD (Fig. 5D).

Apart from these the switch regions and the contact site I, there are, however, other alternative regions of RhoA that should be considered as possible determinants for RID and HR1 binding. Previous studies have shown that at least two regions in RhoA, encompassing amino acids 23–40 and 75–92, are critical for the selective RhoA-ROCK interaction (32, 63). Whereas amino acids 23–40 are integral components of the contact sites I and II (6), amino acids 75–92 constitute a distinct region, which contains 2 asparagines, 87 and 90 in loop 6 (Fig. 5A). Loop 6 of RhoA has been proposed to be essential for determining both binding and cellular functions of ROCK since introduction of a D87V/D90A double mutation in RhoA resulted in a loss of RhoA-ROCK interaction (63). Furthermore, it has been suggested that loop 6 may cooperate with the switch region to promote ROCK activation (63). Another region that might be involved in effector activation process is the insert helix (amino acids 124–136; Fig. 5A) characteristic for Rho family GTPases. Although the function of the insert helix has not been elucidated yet, it has been implicated to be involved in the Rho-dependent activation of ROCK (65) and phospholipase D (66) and in the Rac-dependent p67phox activation (67–69).

The second binding domain (RID) at the N terminus of the RBD is conserved not only in ROCK proteins but also in the Rho effectors kinectin and citron (Fig. 3A). In contrast to ROCK-HR1 but similar to the RBD, ROCK-RID interacts in a GTP-dependent manner with RhoA as it binds RhoA-mantGpp(NH)p with a 4-fold higher affinity than RhoA-mantGDP (Fig. 3, B and C, and Table I). Based on structural and biochemical data, we can exclude the switch regions as the RID-binding site. Although contact site I may be occupied by the HR1 domain of ROCK (see below), a binding at this site by RID cannot be totally excluded. This is particularly due to the possible topological tandem arrangement of RID and RBD similar to the architecture of HR1a and HR1b domains of PRK1. Another RID-binding site might be the region around loop 6, which is quite close to the switch region (Fig. 5A) and thus sensitive to nucleotide-dependent conformational changes.

Unlike the RID-RBD tandem, which is located at the C-terminal region of the amphipathic a-helix, the third Rho-binding module of ROCK (HR1) lies at the N terminus of the predicted coiled-coils and closed to the catalytic kinase domain (Fig. 1A). The fact that ROCK-HR1 shares low homology to both HR1 domains of PRK1 (Fig. 4A) and binds RhoA in a nucleotide-independent manner (Fig. 4, B and C, and Table I) strongly suggests that it approaches RhoA at a different binding site outside the switch regions. Although we can exclude binding to contact site II based on the structure of the RhoA-RBD complex, we cannot rule out the possibility that ROCK-HR1 binds to contact site I based on the homology of the HR1 domain (Fig. 5D).

The HR1 domain of ROCK may therefore play an important role in the structural rearrangement of the kinase domain along the activation process. It may contribute to the release of intramolecular autoinhibition and the subsequent activation of the kinase domain by dimerization and transphosphorylation as suggested in previous studies (35, 70). A region encompassing amino acids 429–647 and 6-553 and containing the HR1 domain of ROCK has been shown to be involved in the dimerization of the kinase (70, 77). According to what has been proposed for myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) and myotonic dystrophy protein kinase (DMPK), two ROCK-related kinases sharing similar topological arrangement and domains, this dimerization domain appears to play a role in regulating kinase activity (71–73). As we found HR1 domains not only in ROCK proteins but also in the coiled-coil regions of other Rho effectors such as citron and kinase (Fig. 4A), this could be an indication for a conserved ROCK-mediated effector activation mechanism.

Although the regulatory mechanism of Rho-mediated effector activation awaits further structural and biochemical investigations, our data presented in this study provided the first detailed biochemical insights into the interaction of Rho with its effectors. Taken together, the presence of multiple effector domains and contact sites on RhoA makes it rather tempting to speculate on two distinct mechanisms: on the one hand, simple recruitment (Rhotekin), and on the other hand, cooperative binding (PRK1 and ROCK) (Fig. 5). In the case of PRK proteins, the HR1a domain recognizes the active GTP-bound state of RhoA and facilitates HR1b binding to contact site I, which in turn may induce a conformational change and thus subsequent activation of the kinase domain (Figs. 1A and 5C). In the case of the ROCK proteins, on the other hand, the dimeric coiled-coil of ROCK-RBD recognizes and interacts with the switch region of two GTP-bound RhoA molecules in a first step (Fig. 5D). In the second step, the RID domains of the ROCK molecules might subsequently approach the loop 6 area of the RhoA molecules, and the resulting structural rearrangement could thereby cancel the autoinhibitory inhibition of the kinase domains. In a third step, the HR1 domains bind to the contact site I of the RhoA molecules and induce the dimerization of the kinase domains of the two ROCK molecules, leading to autophosphorylation and activation.

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