Characterization of RhoA-binding Kinase ROKα Implication of the Pleckstrin Homology Domain in ROKα Function Using Region-specific Antibodies*

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Rho-binding kinase α (ROKα) is a serine/threonine kinase with multiple functional domains involved in actomyosin assembly. It has previously been documented that the C terminus part of ROKα interacts with the N-terminal kinase domain and thereby regulates its catalytic activity. Here we used antibodies against different domains of ROKα and were able to reveal some structural aspects that are essential for the specific functions of ROKα. Antibodies against the kinase domain revealed that this part of the protein is highly complex and inaccessible. Further experiments confirmed that this domain could undergo inter- and intramolecular interactions in a complex manner, which regulates the kinase catalytic activity. Other antibodies that raised against the coiled-coil domain, Rho binding domain, and the pleckstrin homology (PH) domain were all effective in recognizing the native proteins in an immunoprecipitation assay. Only the anti-Rho binding domain antibodies could activate the kinase independent of RhoA. The PH antibodies had no apparent effects on the catalytic activity but were effective in blocking actomyosin assembly and cell contractility. Likewise, mutations of the PH domains can abrogate its dominant negative effects on actin morphology. The subsequent disruption of endogenous ROK localization to the actomyosin network by overexpressing the PH domain is supportive of a role of the PH domain of ROK in targeting the kinase to these structures.

Actin cytoskeleton undergoes rapid dynamic changes in response to extracellular signaling cues, and Rho-family GTPases are key mediators in these responses (1–3). In particular, RhoA is responsible for promoting the formation of actin-based stress fibers and focal adhesions, resulting in contractile phenotype in cultured cells treated with lysophosphatidic acid or sphingosine 1-phosphate (4, 5). Two major effectors of this cytoskeletal event have been identified as ROK1 (ROK/ROCK/ROKα) (6–8) and diaphanous (9, 10), whose cooperative activities upon activation is essential for Rho activities.

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‡ The abbreviations used are: ROK, RhoA binding kinase; PH, pleckstrin homology; CC, coiled coil; BD, binding domain; Ab, antibody; MLC2, myosin light chain 2; HA, hemagglutinin; CAT, catalytic domain; CT, C terminus; GST, glutathione S-transferase; dBD, distal binding domain of ROKα; KIN, kinase.

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autoinhibitory region (20). Binding of the GTP-bound form of Rho is known to activate ROK; the interaction is believed to disrupt the negative regulatory interaction between the kinase domain and the C-terminal autoinhibitory domain to give rise to an active kinase. Although all results have pointed to the C-terminal end of the coiled-coil domain as the Rho-binding site (6, 21–23), the exact sequence of elements involved and nature of the interaction remain largely obscured.

In this current work, we further analyzed the structure and function relationship of ROK. In agreement with the related protein kinases, we found that native ROK exists in multimeric function relationship of ROK. In agreement with the related analysis, the interaction remains largely obscured.

To understand ROK protein kinases, we found that native ROK exists in multimeric function relationship of ROK. In agreement with the related analysis, the interaction remains largely obscured.

In vivo (6)). ROK al. have raised specific antibodies against the various functional domains of ROK as tools to probe its functions in vivo. We show that the antibody against the Rho-binding site could result in ROK activation independent of RhoA. Conversely, the antibody against the C-terminal PH domain produced an inhibitory effect on ROK activity on cell contractility and actomyosin assembly. Combined evidence from immunoprecipitations and mutagenesis studies strongly suggests that the C-terminal PH domain of ROK has a yet undiscovered role on cytoskeleton rearrangement besides its autoinhibitory regulation on the catalytic domain.

MATERIALS AND METHODS

ROK cDNA Cloning and Mutagenesis—HA- and FLAG-tagged full-length wild-type and kinase-dead ROK constructs in mammalian expression vector pGJ40 were obtained as previously described (Leung et al. (6)). ROK-CAT and ROK-kinase-dead constructs (encoding amino acids 1–432) were obtained by PCR with primers 5'-GACTCGAGTCT-TCATCTTTCTG-3' (for the W1331L mutation). The final PCR fragment (encoding amino acids 639–968) were derived from PCR product with primers 5'-GGGTACCCTCTGCGTGGAAGAGAT-3' and 5'-CGGTCTGAGTTCATTTTTTTCCTC-3'. The binding domain ROKα-BD constructs (encoding amino acids 809–1056) were derived from a Hincll/NotI-digested DNA fragment of ROKα and the ROKα-CC constructs (encoding amino acids 1110–1379) were derived from a XmnI DNA fragment of ROKα. ROKα-CT constructs (encoding amino acids 971–1379) were obtained as previously described (6). For generating the ROKα-PH mutant constructs (W1161A/W1313L), a two-step PCR procedure was used with primers 5'-CTTCGGCGCGGATTAAATGTTGTTGATTGTTG-3' (forward) and 5'-GGGCCATCGGGTAGTTCGATTTGAAAAGAAAAG-3'. The resulting construct was subcloned into pGEX-4T1 vector (pBD; encoding amino acids 107–392) was subcloned into pMAL vector. The distal binding domain of ROKα (dBD; encoding amino acids 1008–1139) was obtained by PCR with primers 5'-CGGACAGCTGGGAGAGATCCGCTCGGGAGAGGAGAGAG-3' and 5'-CCCTCGAGATCCGCTCGGGAGAGGAGAGAGAC-3'. The binding domain of ROKβ construct in pGEX-4T1 vector (pBD; encoding amino acids 947–1027) was obtained as previously described (6).

Expression and Purification of Recombinant Proteins and Antibody Purification—Recombinant maltose-binding protein-Rokα-KIN (KIN), GST-ROKα-C1039–968 (CC) ROKα, GST-ROKα142–1779 (PH), and GST-ROKα1027–1379 (pBD) (pBD) were obtained as fusion proteins according to standard protocol. For the preparation of polyclonal antibodies, thrombin-cleaved protein from each GST fusion protein or factor X-cleaved protein from maltose-binding protein fusion protein (400 μg) was emulsified in complete Freund’s adjuvant for injection into rabbits. Rabbits were bled 10 days after the third and subsequent booster injections. Sera were collected and affinity-purified using 2 mg/ml antigen pre-coupled to cyanogen bromide-activated Sepharose (Sigma) and eluted with buffer containing 100 mM glycine-HCl (pH 2.5), 0.05% Triton X-100. The first 2 eluates were neutralized with Tris/HCl (pH 8.5) and used at a 1:1000 dilution for Western blot analysis. Monoclonal antibody 1A1 against the rat ROK kinase domain (39) has been described previously (24, 25). For immunoblotting, Triton-free buffer was used for elution, and the antibody concentrations were adjusted to about 0.5 mg/ml before use.

Preparation of the direct immunoprecipitation or methionine-free MEM medium (Sigma) was supplemented with 10 μCi of [3H]methionine (0.2 μCi/ml; PerkinElmer Life Sciences). Cells were harvested after a 2-h incubation, and sample preparation in lysis buffer and immunoprecipitations were carried out as described before.

ROKα binding was carried out as previously described (6). Briefly, precipitations and mutagenesis studies strongly suggest that the C-terminal PH domain of ROK has a yet undiscovered role on cytoskeleton rearrangement besides its autoinhibitory regulation on the catalytic domain.
Functional Analysis of ROK by Anti-ROKα Antibodies

RESULTS

Specificity of Various Anti-ROK Antibodies in Recognizing the Native Form of ROK—To estimate the endogenous ROK activities in cells and tissues, we have attempted to raise antibodies against the various regions of ROKα for immunoprecipitation assays. An obvious phenomenon from these experiments was the inability of the antibodies against the kinase domain (both monoclonal antibody 1A1 and polyclonal antibodies CC-Ab) in recognizing the native endogenous full-length protein in rat brain extracts (Fig. 1A). Antibodies against the coiled-coil domain of ROKα (CC-Ab), distal RhoA binding domain (BD-Ab), and the PH domain (PH-Ab) were all effective in immunoprecipitating the endogenous ROK (Fig. 1, A and C).

To further investigate the specificity of these antibodies, immunoprecipitations using [35S]methionine-labeled HeLa cell extracts were carried out. Here we showed that except for the kinase antibodies (KIN-Ab), other antibodies (CC-Ab, dBD-Ab, and PH-Ab) could specifically immunoprecipitate both the native and metabolically labeled ROK protein (Fig. 1B).

To see if the anti-kinase domain antibodies are effective in recognizing the kinase domain alone, we used the antibodies to immunoprecipitate the kinase domain overexpressed in transfected COS-7 cells. As shown in Fig. 2A, the overexpressed kinase domain protein was readily immunoprecipitated by the kinase antibody 1A1. Furthermore, the antibody apparently inhibited the catalytic activity of the immunoprecipitated protein. This inhibitory effect could also be observed when 1A1 antibody was co-injected with ROK catalytic domain construct in HeLa cells (Fig. 2B). Injection of this antibody alone into HeLa cells resulted in no obvious effect on the overall actin morphology (Fig. 2B, a and b). However, it was able to almost completely block the aberrant actin filament formation induced by the active ROK-CAT when co-injected (Fig. 2B, c and d), indicating that the antibody had an inhibitory effect against ROK in vivo. These results suggest that the failure of this antibody in recognizing the native full-length ROK protein could be due to inaccessibility of the kinase antibodies to the epitopes.

Native ROK Exists in High Molecular Weight Complexes—It is therefore of great interest to find out why the kinase domain in the full-length protein is inaccessible to the antibodies. We found that full-length ROKα can exist in high molecular weight multimeric forms. As shown in Fig. 3A, endogenous ROKα co-migrated with molecular weight markers in a gel filtration analysis, and no monomeric form of the protein was detectable. A molecular size of about 600 kDa is suggestive of a tetrameric structure. Likewise, chemical cross-linking experiments with bis(sulfosuccinimidyl) suberate also revealed a slow migrating band, confirming that ROK can exist as multimeric complexes (Fig. 3B).

Intra- and Intermolecular Interactions Regulate ROKα Catalytic Activities—To define which region(s) of ROKα is involved in the oligomerization event, we have examined the various regions of ROKα that may have contributed to this event. Both the proximal and distal coiled-coil regions (including the Rho binding domain, which is located at the end of the extended coil regions) were effective in forming oligomers (Fig. 4B). Surprisingly, the kinase domain alone can also form homophilic dimers. In contrast, the PH domain at the C terminus was totally ineffective in dimer formation.

Apart from the intermolecular interaction, we also attempted to determine if intramolecular interactions may also take place. Indeed, the kinase domain can also interact with two independent regions, one at the distal coiled-coil region and an additional interaction at the C-terminal PH domain (Fig. 4C). Hence, our results have revealed that ROKα can form complexes through intermolecular interactions through its coiled-coil and kinase domains and that the N-terminal kinase domain can also interact intramolecularly with the C-terminal coiled-coil and PH domains. To see if these interactions are...
functional, we co-expressed the FLAG-tagged kinase domain with the various HA-tagged interacting partners to check for their associations and effects on the kinase domain activity. As shown in Fig. 4, both the distal coiled-coil and PH domains can partially block the catalytic activities of the co-precipitated kinase domain. Interestingly, dimerization of the wild-type kinase with the kinase-dead ROK/H9251 kinase domain also resulted in an inactive kinase, suggesting that trans-autophosphorylation plays an essential role in regulating the kinase activity.

Antibodies to the Distal Rho Binding Domain Block RhoA Binding and Activate ROK Independent of RhoA—Previous work on ROK isoforms reveal that the Rho binding domain is located at the end of the coiled-coil region (6, 21–23) and that binding of RhoA results in kinase activation (7). Because the antibodies are specifically raised against different domains, we have attempted to determine if binding of these antibodies to the RhoA binding domain of ROK competes with RhoA binding.

As expected, only antibodies against the distal binding domain (dBD-Ab in Fig. 5A) are effective in blocking RhoA binding in vitro. Interestingly, full-length ROK immunoprecipitated with this antibody showed significant increases in the catalytic activity in comparison with ROK protein pulled down by other antibodies (Fig. 5B). This suggested to us that the dBD-Ab not only blocked RhoA binding but also activated ROK catalytic activity.

To further examine the effectiveness of these antibodies in ROK activation in vivo, we have microinjected these antibodies into HeLa cells and checked the effects on actin morphology. As shown in Fig. 5C, cells injected with dBD-Ab gave significant increases in actin stress fibers. These actin filaments were not sensitive to C3 toxin, which is a potent inhibitor of RhoA but is readily disassembled in the presence of the kinase inhibitor HA1077. These results give further support of the in vitro data that the antibodies to the Rho binding domain can activate ROK catalytic activity independent of Rho.

Antibodies against the C-terminal PH Domain Block Cell Contractility and Myosin Assembly—Thus far our results and others clearly show that the C terminus of ROKα can form part of intramolecular interaction with the kinase domain and this can negatively regulate the kinase activity (Fig. 4; Ref. 20). However, although antibodies against the Rho binding domain...
are effective in activating ROKα catalytic activity, the antibodies against the C terminus are apparently ineffective. A consistent observation was the lack of contractility of cells injected with PH-Ab, in contrast to those injected with dBD-Ab (Fig. 6A). Cell contractility was also observed when HeLa cells were treated with Rho-activating agents such as lysophosphatidic acid or sphingosine 1-phosphate (SPP); Fig. 6B). In contrast, the responsive-ness to these agents was lost when cells were injected with PH-Ab (Fig. 6A). To further characterize this phenotypic effect upon PH-Ab, we have stained injected cells for myosin light chain, polymerized actin filaments, and focal adhesions. As shown in Fig. 6B, cells injected with PH-Ab exhibited a prominent defect on myosin assembly with moderate changes in polymerized actin arrangement (Fig. 6B, a and b). By contrast, cells injected with dBD-Ab showed marked enhancement in myosin staining, whereas CC-Ab was relatively ineffective (Fig. 6B, c and d). PH-Ab-injected cells also showed mild but consistent decreases in focal adhesion staining (Fig. 6C, a and b) in
contrast to cells injected with dBD-Ab, which showed marked increases (c and d), and to those with CC-Ab, which remained unchanged (e–f). Thus, PH-Ab apparently exerted a potent effect on myosin assembly with relatively milder effects on actin and focal adhesions. These cytoskeletal effects of the PH-Ab may account for the observed lack of contractility in

FIG. 6. Antibodies to the PH domain blocks cell contractility and myosin assembly. A, HeLa cells in serum medium were injected with anti-Rho-binding domain antibody dBD-Ab (a) or anti-PH domain antibody PH-Ab (b) (arrows, top panel). Phase contrast images were taken after 30 min after injection. For sphingosine 1-phosphate (SPP) treatment, serum-starved HeLa cells were injected with PH-Ab and incubated for 30 min before SPP treatment. Time lapse on the phase contrast images were followed and taken at 0 time and 15 min after treatment (bottom panel). B, HeLa cells were injected with the various anti-ROKα antibodies (PH-Ab, dBD–Ab, and CC-Ab) and incubated for 30 min before fixing and staining with anti-myosin antibody (a, c, and d) and TRITC-phalloidin (b). Arrows mark the injected cells. C, HeLa cells were injected with PH-Ab, dBD-Ab, and CC-Ab as in B and stained with mouse anti-vinculin antibody. Fluorescein isothiocyanate-anti rabbit IgG was used to show the injected cells (arrows).
PH-Ab-injected cells upon sphingosine 1-phosphate treatment.

**PH Domain of ROKα Has Multiple Functional Roles**—The observation of an inhibitory effect of the PH-Ab has prompted us to investigate if the C-terminal PH domain of ROKα may have other functional roles apart from its intramolecular kinase inhibition. A mutant with two conserved tryptophan residues (ROKα-CT^AL; Refs. 25 and 26) was used to examine its effects on kinase inhibition. As shown in Fig. 7A, this mutant was as effective as the wild-type protein in interacting and inhibiting the kinase domain catalytic activity (Fig. 7A). However, the dominant negative effect of this mutant protein on actin cytoskeleton is lost (Fig. 7B), suggesting that the PH domain may have a role that is unrelated to the intramolecular kinase inhibition. Because both PH-Ab and overexpression of minimal PH domain apparently had similar cytoskeletal effects, we attempted to examine the effect of the PH domain on the localization of the endogenous ROK protein in Swiss 3T3 fibroblasts using the CC-Ab. As shown in Fig. 7C, interphase fibroblast showed endogenous ROK co-localized with myosin filaments (a and b), in agreement with recent data showing co-localization of Rho regulatory proteins in these structures (27). When overexpressing the PH domain, the distribution of endogenous ROK was more diffused, and myosin arrangement was in a random fashion (c and d). Expression of the mutant PH domain (ROKα-PH^AL) apparently had no such effect, and endogenous ROK showed the normal close alignment with assembled myosin. Similar effects were observed with a PH domain mutant at the cysteine-rich domain region within the PH domain (C1284S/C1287S; data not shown). We therefore conclude that an intact PH domain is required for the blockage of endogenous ROK protein to localize to the site of myosin assembly.

**DISCUSSION**

We have described in this report that antibodies against the kinase domain of ROKα can recognize the kinase domain alone but not the full-length protein. Further experiments have indicated that the kinase can exist in multimeric form, as the result of extensive intermolecular interactions of the various coiled-coil domains. A tetrameric structure can be predicted based on the molecular size of about 600 kDa on gel filtration chromatography and chemical cross-linking. Both the proximal and distal coiled-coil domains can form independent homophilic dimers. Furthermore, the C terminus can also interact intramolecularly with the kinase domain. These results are...
similar to an earlier report (20), but our data suggest that a major interaction also include the distal coiled-coil region that overlaps with the RhoA binding domain, although the C-terminal PH domain may also contribute to the overall effect. Interestingly, the kinase domain is also able to form homodimers. The myotonic dystrophy kinase family including ROKα consists of a conserved activation loop and an extended C-terminal hydrophobic loop that are known for catalytic activity (15–17). In some cases the phosphorylation within these two regions can be achieved by other kinases or through an autocatalytic event. Our results of an inactive ROKα kinase when an active kinase domain dimerized with a kinase-dead counterpart may suggest that a trans-autophosphorylation event upon kinase dimerization is essential for kinase activation. The observation that autophosphorylation site mutants ROKα^T240^A in the activation loop region and ROKα^T405^A in the extended hydrophobic region that exhibited significant losses of catalytic activity also provides further support of this regulatory event (data not shown). Furthermore, co-expression of wild-type ROKα with PDK1 (both active and kinase-dead forms) did not result in alterations in ROKα catalytic activity, implying that ROKα is not regulated by this kinase (data not shown). It is tempting to speculate that the dimerization and autophosphorylation of ROKα may well be a major event for the regulation of these kinases. Our recent data on the related Cdc42-binding kinase myotonic dystrophy kinase-related Cdc42-binding kinase-a has also provided strong evidence for this notion. In this case the dimerization and activation of the kinase domain (which depends on the conserved N-terminal extended region) and the inhibitory interaction between the kinase domain and the distal coiled-coil domain are mutually exclusive and crucial for regulating the catalytic activity (18). A similar conserved mechanism may also be involved in the dimerization of the kinase domain of ROKα and its distal coiled-coil domain, with an additional requirement that a larger C-terminal region is required for effective interaction and kinase inhibition (20). We therefore conclude that ROKα can form multimeric complexes essentially with the central coiled-coil region. The homophilic dimer formation of the kinase domain is crucial for the catalytic activity, and this can be abrogated by interaction with the C-terminal region, in particular the distal coiled-coil region, which overlaps with the Rho-A binding domain and forms an essential part of the autoinhibitory motif.

In this respect, it is intriguing to find that antibodies to the Rho binding domain can activate ROK kinase activity. It is conceivable that the interaction of the antibody with an exposed surface at this region close to the Rho binding and the overlapping kinase inhibitory interaction region is sufficient to cause a conformational change leading to activation of the kinase in a Rho-independent manner. We have previously mapped the Rho binding domain of ROKα to residues 968–1047 (6), and more recent work (21–23) has documented a more likely binding site at a nearby N-terminal region. Antibodies directed specifically to the Rho-binding site of ROKβ were ineffective in recognizing the protein, indicating this region may not be exposed and, hence, inaccessible to the antibodies (see Fig. 1). The requirement of antibodies against an extended region beyond the Rho binding domain for the recognition and activation of ROKα may imply multiple binding sites for Rho. A weaker site at the C-terminal side of the Rho binding region may be necessary for opening up the distal coiled-coil region, where a more pertinent Rho binding can occur at a subsequent stage. It is likely that such perturbation may be crucial for releasing the inhibitory effect of this region upon the kinase domain, whose subsequent dimerization described earlier is essential for kinase activation.

Although the PH domain can also form part of the autoinhibitory domain, it is not known whether it can serve other functional roles because PH domains are known to interact with phospholipid and membrane components (28). Interestingly, the PH domain of ROKα is unique, consisting of an internal cysteine-rich motif. Previous reports indicate that this motif alone can block stress fibers and focal adhesion formation, probably through the autoinhibitory effect on the kinase catalytic activity (6, 20). However, our observation that the anti-kinase antibodies are unable to recognize the full-length protein may suggest that overexpression of the PH domain may not have major effects on the full-length protein because this could only be effective intramolecularly. Support of this view comes from the microinjection experiments with the PH antibodies that resulted in defective cell contractility and myosin assembly. Furthermore, PH mutants at the PH/cysteine-rich domain regions are significantly less effectively in blocking actin stress fiber and focal adhesion formation in comparison with the wild-type PH domain, although they still retained the ability to interact with the kinase domain protein and inhibited the catalytic activity. This provides strong evidence that this region of ROKα may have an additional functional role. The observation of a blockage of translocation of endogenous ROK to myosin assembly site may imply that this region of ROKα may play a role in kinase targeting to and subsequent assembling myosin.

In summary, we have raised antibodies to the various regions of ROKα. Antibodies against the kinase domain have revealed that this part of the kinase is cryptic. This phenomenon probably the result of extensive inter-and intramolecular interactions of the different domains that also form part of the kinase activation mechanism. Antibodies to the Rho binding domain can essentially replace Rho in terms of kinase activation, and antibodies to the C-terminal PH domain also revealed that this part of the kinase is essential for targeting of the kinase to actomyosin compartment. These antibodies are therefore useful in defining functions to the various structural domains of the protein.

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