Analysis of RhoA-binding Proteins Reveals an Interaction Domain Conserved in Heterotrimeric G Protein β Subunits and the Yeast Response Regulator Protein Skn7*

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To identify potential RhoA effector proteins, we conducted a two-hybrid screen for cDNAs encoding proteins that interact with a Gal4-RhoA.V14 fusion protein. In addition to the RhoA effector ROCK-I we identified cDNAs encoding Kineticin, mDia2 (a p140 mDia-related protein), and the guanine nucleotide exchange factor, mNET1. ROCK-I, Kineticin, and mDia2 can bind the wild type forms of both RhoA and Cdc42 in a GTP-dependent manner in vitro. Comparison of the ROCK-I and Kineticin sequences revealed a short region of sequence homology that is both required for interaction in the two-hybrid assay and sufficient for weak interaction in vitro. Sequences related to the ROCK-I/Kineticin sequence homology are present in heterotrimeric G protein β subunits and in the Saccharomyces cerevisiae Skn7 protein. We show that β2 and Skn7 can interact with mammalian RhoA and Cdc42 and yeast Rho1, both in vivo and in vitro. Functional assays in yeast suggest that the Skn7 ROCK-I/Kineticin homology region is required for its function in vivo.

Members of the Rho family of GTPases regulate diverse cellular processes ranging from cytoskeletal organization to gene expression and cell transformation. Upon binding GTP, these Ras-like proteins interact with effector proteins to induce downstream signals (for reviews see Refs. 1–3). Recent biochemical and genetic studies have identified many potential Rho effectors in both mammalian cells and the budding yeast Saccharomyces cerevisiae. Mammalian RhoA interacts with members of the PKN/PRK and ROCK/ROK protein kinase families (4–10). The ROCKs are clearly involved in cytoskeletal rearrangements (6, 11–15), but the functions of the PKN/PRK kinases remain obscure. RhoA also interacts with several apparently non-catalytic effectors including Rhophilin, Rhotekin, Citron, the myosin-binding subunit of myosin light chain phosphatase (MBS),1 p140 mDia, Kineticin, and p116RIP (9–11, 14–18). RhoA interactions with MBS, p140 mDia, and Kinectin are likely to be involved in contractile events, actin polymerization and cytokinesis, and motility, respectively (11, 17, 19, 20). In S. cerevisiae, RHO1, an essential gene (21, 22), controls activity of 1,3-β-glucan synthase, the enzyme that synthesizes cell wall glucan polymers (23, 24), and BNI1, a gene involved in cytoskeletal events and cytokinesis (25, 26). Rho1 regulates the PKC1-MPK1 MAP kinase pathway that controls cell wall integrity (27, 28) and the Rim1 transcription factor (29, 30). Interestingly, Skn7, a yeast two-component protein, shows genetic interactions with the PKC1 pathway and exhibits several properties that suggest that it too may be a Rho effector (31–33).

Sequence elements involved in the interaction between Rho family GTPases and their effectors are of considerable interest since their definition should permit the identification of further potential effector proteins. The first such motif to be identified was the CRIB (Cdc42/Rac interactive binding) motif, which specifies interaction with Rac1 and Cdc42, but not RhoA, and has the consensus sequence ISXXPXX2 or 3FXHXX2(H/T)(V/A)(G/D/Q) (34). A second motif, REM-1 (Rho effector motif class 1), is found in the N-terminal Rho-binding domains of the RhoA effectors PKN/PRK1, PRK2, Rhotekin, and Rhophilin and has the consensus (L/I/F/X2(E/K)/X3(7)G/2(V/A)(E/R)(N/R/K) (9, 14). REM-1 appears specific for RhoA, although PRK2 has also been reported to bind Rac1 (8). Although the REM-1 motif is reiterated three times at the N terminus of the PKN/PRK kinases, only a single copy is found in Rhophilin and Rhotekin. REM-1 motifs appear to differ in their ability to discriminate between the GTP- and GDP-bound forms of RhoA.2 No sequence motifs common to other RhoA effectors have been identified, although the defined Rho-binding regions of Citron, Kineticin, and the ROCKs are all found in regions of extended α-helical coiled-coil structure.

To identify potential RhoA effectors, we conducted a two-hybrid screen with RhoA.V14 as a bait. In addition to ROCK-I, we identified cDNAs encoding Kineticin, a p140 mDia-related protein, and the mouse homolog of the NET1 GEF. Comparison of the ROCK-I and Kineticin sequences revealed a short region of sequence homology that is both required for and sufficient for interaction with RhoA and Cdc42. A similar sequence is found in both heterotrimeric G protein β subunits and the yeast Skn7 protein and is also required for the interaction of these proteins with RhoA. In addition to defining a RhoA interaction domain, the ROCK-I/Kineticin homology region is required for the func-

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1 The abbreviations used are: MBS, myosin-binding subunit; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GTP-γS, guanosine 5′-O-(thiotriphosphate); GEF, guanine nucleotide exchange factor.

2 Flynn, P., Mellor, H., Palmer, R., Panayotou, G., and Parker, P. J. (1998) J. Biol. Chem. 273, 2698–2705.

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tion of Skn7 in vivo, consistent with the notion that Skn7 represents a novel Rho effector.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids were constructed by standard techniques; details are available on request. The SKN7 (GenBank™ accession number U90465; Ref. 31) and human β2 subunit (GenBank™ accession number M16538; Ref. 35) coding sequences were obtained by PCR and their DNA sequences confirmed.

GAL4 DNA-binding Domain Fusions—Activated RhoA and Cdc42 were subcloned from mammalian expression plasmids (36) as Ncol + Xho fragments into pGPTB9; the RhoA CAAX motif was inactivated by a C190S mutation introduced by PCR and that of Cdc42 by the strategy generating Cdc42(1–178)-ID. Yeast RhoA was isolated using PCR and Rho1.G19V/C206S constructed by standard techniques.

GAL4 Activation Domain Fusions—cDNA clones isolated in the two-hybrid screen are summarized in Table I. For further two-hybrid analyses DNA fragments were inserted into derivatives of pGAD424 and pGAD10 (CLONTECH). A cDNA encoding a CRIB domain protein related to MSE55 (34) was used as a specificity control in the two-hybrid assay. The plasmids encode the following sequences C-terminal to the Gal4 activation domain. cDNA clone D1 encodes IWSNPDREFT (ROCK-I codons 303–1030)-KKKVNSRDL. cDNA clone D4 encodes IWSNDRPNNPSSP-(ROCK-I codons 456–1028)-VNLERSMNRYY.

cDNA clone D9 encodes IWSNPDREFT-(ROCK-I codons 349–1025)-GELEMRNSRYY. GAL4-ROCK-(831–1010) encodes IEFPM-(ROCK-I codons 831–1010)-RHOFPYMN. GAL4-ROCK-(831–1010)/TT is the same as GAL4-ROCK-(831–1010) but with K1005L/T1006T. GAL4-ROCK-(831–1010)-AHR encodes ISGRSG-(ROCK-I codons 831–1010)-FQIYEV with codons 950–966 deleted. GAL4-ROCK.HER encodes ISGRSG-(ROCK-I codons 950–972)-FQIYEV. GAL4-Kinectin-(1053–1327) encodes IWSNPDREFT-(cDNA clones D2 and D3) and encodes IWSNDRPNNPSSP-(ROCK-I codons 1053–1327). GAL4-Kinectin-(1053–1327)AHR encodes IEFPMGRDLP. (Kinectin codons 1053–1327) with codons 1191–1215 replaced by GS, GAL-mDia2(24–275) (cDNA clone D7) encodes mDia2 IWSNPDREFT-(mDia2 codons 47–800)-VNSREIYEV. GAL4-NET1 (clone D5) encodes IWSNDRPNNPSSP-(cDNA fragments into pGBT9; the RhoA CAA motif was inactivated by a C190S mutation introduced by PCR and that of Cdc42 by the strategy generating Cdc42(1–178)-ID. Yeast RhoA was isolated using PCR and Rho1.G19V/C206S constructed by standard techniques.

Two-hybrid Screen—Yeast strains and manipulation procedures involved in the two-hybrid screen are summarized in Table I. For further two-hybrid analyses DNA fragments were inserted into derivatives of pGAD424 and pGAD10 (CLONTECH). A cDNA encoding a CRIB domain protein related to MSE55 (34) was used as a specificity control in the two-hybrid assay. The plasmids encode the following sequences C-terminal to the Gal4 activation domain. cDNA clone D1 encodes IWSNPDREFT (ROCK-I codons 303–1030)-KKKVNSRDL. cDNA clone D4 encodes IWSNDRPNNPSSP-(ROCK-I codons 456–1028)-VNLERSMNRYY.

cDNA clone D9 encodes IWSNPDREFT-(ROCK-I codons 349–1025)-GELEMRNSRYY. GAL4-ROCK-(831–1010) encodes IEFPM-(ROCK-I codons 831–1010)-RHOFPYMN. GAL4-ROCK-(831–1010)/TT is the same as GAL4-ROCK-(831–1010) but with K1005L/T1006T. GAL4-ROCK-(831–1010)-AHR encodes ISGRSG-(ROCK-I codons 831–1010)-FQIYEV with codons 950–966 deleted. GAL4-ROCK.HER encodes ISGRSG-(ROCK-I codons 950–972)-FQIYEV. GAL4-Kinectin-(1053–1327) encodes IWSNPDREFT-(cDNA clones D2 and D3) and encodes IWSNDRPNNPSSP-(ROCK-I codons 1053–1327). GAL4-Kinectin-(1053–1327)AHR encodes IEFPMGRDLP. (Kinectin codons 1053–1327) with codons 1191–1215 replaced by GS, GAL-mDia2(24–275) (cDNA clone D7) encodes mDia2 IWSNPDREFT-(mDia2 codons 47–800)-VNSREIYEV. GAL4-NET1 (clone D5) encodes IWSNDRPNNPSSP-(cDNA fragments into pGBT9; the RhoA CAA motif was inactivated by a C190S mutation introduced by PCR and that of Cdc42 by the strategy generating Cdc42(1–178)-ID. Yeast RhoA was isolated using PCR and Rho1.G19V/C206S constructed by standard techniques.

In Vitro Binding Assay—Equal amounts of each GST-fusion protein (~100–300 ng) were bound to glutathione-Sepharose beads and incubated with 10 ng of GTPγS- or GDP-loaded RhoA.WT-9E10 or Cdc42.WT-9E10 at 4 °C for 2 h, with agitation, in RB containing 0.5 mg/ml bovine serum albumin. The beads were then washed in ice-cold RB, 0.1% Nonidet P-40, and bound GTPase was eluted by boiling in SDS-PAGE sample buffer. Following fractionation by SDS-PAGE, GTPases were visualized by autoradiography or by comparison to known standards on Coomassie-stained SDS-polyacrylamide gels.

RESULTS

Identification of Potential RhoA Effectors by the Two-hybrid Screen—We performed a two-hybrid screen for proteins that can interact with the activated form of RhoA. A fusion gene, Gal4-RhoA(V14/S190), was constructed in which the Gal4 DNA-binding domain is fused N-terminal to activated human RhoA (RhoA.V14), carrying an additional mutation at its C terminus to destroy the CAAX motif. Yeast HF7c cells expressing Gal4-RhoA(V14/S190) were used to screen a library of Gal4 activation domain-tagged mouse T-cell cDNA. Seven transformants exhibited Gal4-RhoA(V14/S190)-dependent activation of both the HIS3 and LacZ markers in HF7c cells. The cDNAs were characterized by partial DNA sequencing (Table I). Plasmids D1, D4, and D9 carry partial cDNAs encoding the RhoA effector kinase ROCK-I (p160ROCK; Ref. 7). Plasmid D2 and D3 carried the same partial Kinectin cDNA (20); interestingly, although Kinectin was previously identified as a putative RhoA effector, the cDNA fragment isolated in our screen does not overlap that isolated in the previous screen (15; see “Discussion”). Plasmid D5 carried a complete open reading frame 83% identical to the putative Rho family guanine nucleotide exchange factor NET1 (40); functional characterization of this protein will be published elsewhere.

Plasmid D7 contains a cDNA related to p140 Dia, as identified in a previous screen for RhoA effector proteins (17) which is related to the Drosophila gene Diaphanos (41). In vitro experiments described below indicated that the RhoA-binding domain of this protein is located in its N-terminal sequences; we therefore sequenced this region, which spans codons 47–257, and compared it with p140 Dia and with Drosophila Diaphanos (Fig. 1). A full characterization of this protein, to which we refer as mDia2, is currently in progress.

RhoA and Cdc42 Bind to Kinectin, ROCK-I, mDia-2 in a

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TABLE I 
cDNAs recovered in two-hybrid screen

| cDNA | Identitya | Codonsb |
|------|-----------|---------|
| D1   | Mouse ROCK-I (U58512) | 300–1030 |
| D4   | 456–1028 | |
| D9   | 349–1025 | |
| D2   | Mouse Kinectin (L43326) | 1053–1327 |
| D3   |  | |
| D5   | Mouse NET1 | 1–595 |
| D7   | Dbl domain bearing Rho family GEF | 47–800 |

a GenBank™ accession numbers are given in parentheses.

b Residues encoded by each insert are shown. The same Kinectin cDNA was identified in two independent transformants.

1 The N-terminal residue of the mDia2 sequence is assigned by comparison with a mouse EST (GenBank™ accession number AA415402; Fig. 1) and its C-terminal residue by comparison with the p140 Dia sequence. Full details of the mDia2 cDNA clone will be published elsewhere.

2 bin was removed by adsorption to p-aminoenobenazamide-Sepharose 6B (0.5 ml; Sigma). Protein concentrations were determined by dye-binding assay (Bio-Rad) or by comparison to known standards on Coomassie-stained SDS-polyacrylamide gels.

3 A. S. Alberts and R. Treisman, unpublished data.

4 A. S. Alberts and R. Treisman, manuscript in preparation.
GTP-dependent Manner—We used the two-hybrid assay to investigate the interactions between RhoA, its yeast homolog Rho1, and human Cdc42 and the proteins identified in the screen, in each case using Gal4-GTPase fusion proteins containing activating mutations and mutated CAAX motifs. Each of the three GTPases could interact with all the proteins in the assay (Fig. 2A). To confirm the specificity of the interactions with Cdc42, we examined interaction of an MSE55-related protein isolated in a screen for Cdc42 effectors; this CRIB motif-containing protein interacted with Cdc42.V12, but not RhoA.V14, in the two-hybrid assay in agreement with previous results (Fig. 2A; Ref. 34).

The two-hybrid data suggest that each of the cDNAs isolated in the screen encodes a protein that can interact with activated forms of both RhoA and Cdc42. To confirm that the wild type GTPases can also interact with these proteins and to investigate whether binding is GTP-dependent, we performed in vitro binding assays. GST fusion genes carrying each potential Rho effector were constructed as follows: Kinectin-(1053–1327), ROCK-I-(831–1010), mDia2-(47–257), and mNET1-(1–595). To assess the specificity of the assay, we also tested the fusion proteins PKN-(1–511) and PAK-(1–252) which specifically bind RhoA and Cdc42, respectively (9, 42). Recombinant 9E10 epitope-tagged RhoA and Cdc42 proteins were purified from bacteria, loaded with either GTP\(_{\gamma}\)S or GDP, and incubated with equimolar amounts of the various GST fusion proteins. Following washing, bound GTPases were detected by immunoblot with the 9E10 antibody (Fig. 2B). Binding of PKN-(1–511) and PAK-(1–252) was specific for the GTP-bound forms of RhoA and Cdc42, respectively, demonstrating that our assay conditions allow discrimination between the two GTPases (Fig. 2B, lanes 13–16). The RhoA-binding regions from Kinectin, ROCK-I, and mDia2 bound both wild type RhoA and wild type Cdc42 in a GTP-dependent manner (Fig. 2B, lanes 5–10). In contrast, although binding of mNET1 to Cdc42 was GTP-dependent, it bound RhoA.GTP and RhoA.GDP equally well (Fig. 2B, lanes 11 and 12). Taken together with the two-hybrid data, these results show that binding, ROCK-I, and mDia2 all represent potential effectors for both RhoA and Cdc42; we show elsewhere that mNET1 is a RhoA GEF (see “Discussion”).

mROCK-I and Kinectin Have Similarities within Their Rho-binding Domains—The RhoA effectors Rhotekin, Rhophilin, and PKN share a region of homology within their Rho-binding domains (the REM-1 motif; 14). Previous studies of the ROCK proteins have defined a short region within its C-terminal coiled-coiled region that suffices for interaction with RhoA (4, 5, 43). We therefore compared the sequence spanning this region with the sequences of Kinectin, mDia2, and mNET1 to identify potential Rho-binding sequence motifs. Although the maxi-

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5 A. S. Alberts, unpublished data.
The ROCK-I/Kinectin Homology Is Required for Interaction with Rho Proteins—To investigate the significance of the ROCK-I/Kinectin homology region, we examined its role in the interactions with RhoA and Cdc42 using both two-hybrid assays (Fig. 4) and in vitro biochemical assays (Fig. 5). To facilitate mutagenesis of ROCK-I, we examined a shorter ROCK-I fragment containing codons 831–1010, which interacts strongly with RhoA.V14 and Cdc42.V12 in the two-hybrid assay (Fig. 4A, lanes 1 and 2) and with GTP-loaded RhoA and Cdc42 in the in vitro binding assay (Fig. 5A, lanes 5). Sequences encompassing the homology were deleted from both ROCK-I and Kinectin, and the interactions of the resulting proteins with RhoA and Cdc42 were examined. Deletion of the homology region (codons 950–972) from ROCK-I-(950–972) severely impaired its interaction with RhoA in the GTPase binding assay, and all interactions were abolished by deletion of the ROCK-I/Kinectin homology region (Fig. 5A, lanes 7–10). Neither protein interacted with GDP-bound GTPases (data not shown). Thus, both β2 and Skn7 interact with GTP-bound Rho proteins.

The Homology Region Is Sufficient for Interaction with RhoA or Cdc42—We next tested whether the ROCK-I/Kinectin homology region is sufficient for binding to RhoA and Cdc42. Sequences encompassing the homology from ROCK (codons 950–972) or β2 (codons 1–32) were inserted into appropriate plasmids for use in the two-hybrid and in vitro interaction assays. In addition to deletion of the ROCK-I/Kinectin homology region, previous studies have demonstrated that point mutations of ROK-α (ROCK-II) residues C-terminal to the homology severely impair its interaction with RhoA in GTPase overlay assays (5). We therefore constructed an analogous mutant, ROCK-I-(831–1010)-TT and compared its binding properties with those of the isolated ROCK-I/Kinectin homology region.

In the two-hybrid assay ROCK-I-(950–972), the isolated ROCK homology region peptide interacted with both RhoA.V14 and Cdc42.V12, as did ROCK-I-(831–1010)-TT (Fig. 4A, rows 4 and 5). The N-terminal region of β2 was also sufficient for interaction with both GTPases in this assay (Fig. 4A, row 12). Similar results were obtained in the in vitro binding assay. GST fusion derivatives carrying ROCK-I-(950–972), the isolated ROCK homology region peptide, and ROCK-I-(831–1010)-TT interacted weakly with both GTPases, whereas deletion of the ROCK-I/Kinectin homology reduced interaction to background levels (Fig. 5B, lanes 3–6). A GST derivative carrying β2-(1–32) was sufficient for interaction with both GTPases in vitro (Fig. 5B, lanes 7–9). Control experiments with PKN-(1–511) and PAK-(1–252) confirmed that the assay conditions allow discrimination between RhoA- and Cdc42/Rac-specific effector proteins (Fig. 5B, lanes 10 and 11). Taken together, these data suggest that the ROCK-I/Kinectin homology region mediates interactions with these GTPases (see “Discussion”).

The ROCK-Kinectin Homology Region Is Required for Skn7 Function—The results presented in the preceding section provide strong evidence that Skn7 interacts with both RhoA and Rho1 and show that the ROCK-I/Kinectin homology region is required for this interaction. We therefore examined the role of the ROCK-I/Kinectin homology in Skn7 function in yeast using a number of different assays (Table II). These assays rely either...
on measuring the effects of Skn7 overexpression in different genetic backgrounds or on measuring the ability of different Skn7 mutants to suppress the effects of SKN7 deletions. High level overexpression of SKN7 from the GAL1 promoter in wild type cells is lethal, probably owing to weakening of the cell wall (33). Skn7 overexpression from high copy number plasmids also activates the MCB promoter element which is partly responsible for G1 cyclin gene expression; Skn7 overexpression can therefore bypass the normal requirement for the SWI4 and SWI6 gene products, allowing growth of swi4ts swi6 cells at the nonpermissive temperature (33). In addition, Skn7 overexpression partially suppresses the temperature-sensitive phenotype of cells expressing human RhoA, allowing them to grow at 35.5 °C; in contrast, it exacerbates the severity of the temperature-sensitive cdc42 mutation, preventing growth at 35.5 °C (33).

Deletion of SKN7 has a number of effects, rendering cells acutely sensitive to oxidative stress (44) and preventing growth of pkc1-8 cells at 37 °C (32, 33, 37). Moreover, deletion of SKN7 prevents suppression of the swi4ts swi6Δ double mutation by Mbp1 overexpression (7). For these studies we constructed a yeast expression plasmid carrying a derivative of the full-length SKN7 open reading frame, Skn7ΔHR, which lacks residues 237–259, spanning the ROCK-I/Kinectin homology region. As controls we examined wild type Skn7 and two other mutants that affect Skn7 function. One, Skn7D427N, lacks the phosphoacceptor aspartate in the receiver domain which is essential for Skn7 function in the cell cycle (33) but not in the response to free radical stress (44). The other, Skn7Δ-(353–623), contains a C-terminal deletion that inactivates the protein (8). Each of the mutant proteins could be detected by immunoblotting of yeast cell extracts with Skn7 antiserum, so defects in their function cannot be attributed to changes in protein stability (data not shown).

The Skn7D427N and Skn7Δ-(353–623) mutants behaved exactly as expected in all the assays (Table II, lines 2 and 4). The Skn7ΔHR protein was active in one of the assays tested, preventing growth of cdc42 at 35.5 °C (Table II, line 2). This result

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**Fig. 4.** The ROCK-I/Kinectin homology is necessary and sufficient for two-hybrid interactions with RhoA, V14, and Cdc42.V12. A, analysis of different effector protein mutants. Proteins lacking the ROCK-I/Kinectin homology region are denoted ΔHR. Interaction strength was assessed by growth on histidine-selective medium containing increasing amounts of 3-aminotriazole. Scores 1, 2, 3, 4, and 5 correspond to growth on plates containing 0, 1, 2, 4, or 8 mM 3-aminotriazole, respectively. B, plate growth assay for interactions. HF7C yeast were transformed with the indicated combinations of plasmids expressing GAL4-DNA-binding domain/GTPase or Gal4 activation domain/effector fusion proteins. Two independent transformants in each case were assayed for growth after 3 days at 30 °C on selective medium lacking histidine and containing 2 mM 3-aminotriazole.

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**Fig. 5.** A, the ROCK-I/Kinectin homology is necessary for in vitro interactions with RhoA, V14, and Cdc42.V12. 9E10-tagged wild type RhoA (top panel) or Cdc42 (bottom panel) were preloaded with either GTPyS or GDP. Each GTPase (10 ng) was incubated with glutathione beads carrying equimolar amounts of the indicated GST-effector fusion proteins. Proteins lacking the ROCK-I/Kinectin homology region are denoted ΔHR. Following washing, bound GTPases were eluted and fractionated by SDS-PAGE. GTPases were detected by immunoblotting using the 9E10 antibody. Load, total GTPase input protein. B, the ROCK-I/Kinectin homology is sufficient for in vitro interactions with RhoA, V14, and Cdc42.V12. In vitro binding assays were performed as in A with the indicated GST fusion proteins.

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6 N. Bouquin and L. H. Johnston, unpublished data.
7 N. Bouquin and L. H. Johnston, manuscript in preparation.
8 N. Bouquin, unpublished data.
Novel Rho Family Effector Proteins

Table II
The ROCK/Kinectin homology is required for SKN7 function

| Gene* | pGAL overexpression§ | swi6Δ | p3MBC-lacZ| swi6Δ | RhOA | cdc42Δ | H2O2§ | pkc1–8 | swi6Δ | skn7Δ | Yep MBP1? |
|-------|----------------------|-------|-----------|-------|-------|--------|--------|--------|-------|--------|----------|
| 1. SKN7 | Lethal | + | + | + | + | + | + | + | + | ND |
| 2. skn7ΔD427N | Lethal | + | + | – | ND | + | + | + | + | ND |
| 3. skn7ΔHR | Viable | + | – | – | – | – | – | + | – | – |
| 4. skn7Δ533–623 | ND | – | – | – | – | – | – | – | – | – |

* SKN7, intact wild type gene; Skn7ΔD427N, mutation of the aspartate phosphatase receptor in the receptor domain (32, 33); Skn7ΔHR, deletion of the ROCK-I/Kinectin homology region (32–260); Skn7Δ533–623, deletion of the C-terminal deletion of SKN7.

§ Reporter gene assay with a lacZ reporter controlled by three MCB elements. High copy SKN7 activates this reporter in a swi6Δ genetic background (33), +, blue color.

Suppression of the swi4ts swi6Δ strain K2003 which is temperature-sensitive for growth at 37 °C. High copy SKN7 suppresses this by stimulating G1 cyclin gene expression (33). +, growth at 37 °C.

Strain YOC725 expressing human RhOA. High copy SKN7 weakly suppresses the temperature-sensitive growth phenotype of this strain. +, growth at 35.5°C.

Cells of the cdc42–1 mutant (kindly provided by John Pringle) do not grow at 37 °C but will grow at 35.5 °C. High copy SKN7 antagonizes this effect and prevents growth at 35.5 °C. +, growth at 35.5 °C; –, no growth at 35.5 °C.

Skn7Δ cells are acutely sensitive to H2O2 (44). +, suppression of sensitivity.

pkc1–8 is a temperature sensitive pkc1 mutant that grows poorly at 37 °C (37) but is lethal in combination with skn7Δ (32, 33). Single copy SKN7 derivatives were expressed and scored for growth at 37 °C (+).

swi4ts swi6Δ cells are temperature-sensitive for growth at 37 °C but can be suppressed by high copy expression of MBP1, provided functional SKN7 is present. +, growth at 37 °C.

ND, not determined.

demonstrates that the ROCK-I/Kinectin homology region is not required for this aspect of Skn7 function and serves as a positive control since it confirms the structural integrity of the Skn7ΔHR protein. In sharp contrast, the Skn7ΔHR mutant was inactive in all the other assays examined (Table II). Thus, the ROCK-I/Kinectin homology region is essential for the bulk of Skn7 function in vivo as well as its interaction with RhoA and RhOA.

Discussion

In this work we used a two-hybrid screen to identify potential effector proteins of the mammalian Rho family GTPase RhOA. We identified cDNAs encoding two previously characterized effectors, ROCK-I and Kinectin, together with cDNAs for mNET1, the mouse homolog of NET1 (40), a putative guanine nucleotide exchange factor (GEF), and a novel protein, mDia2, which is related to p140 mDia (17). Our Kinectin cDNA spans codons 1053–1327, a region that is distinct from that identified as a RhOA-binding domain in a previous two-hybrid screen (15), which suggests that the protein contains multiple RhO-binding elements. Although the ROCK-I, Kinectin and mDia2 proteins are bound to both RhOA and Cdc42 in a GTP-dependent manner, mNET1 exhibited similar affinities for both GTP- and GDP-bound RhOA. This behavior was not unexpected because the protein contains a Dbl homology domain, associated with RhoA weakly in vitro suggests that it makes direct contacts with the GTPase. However, previous studies of ROCK-I have shown that point mutations or deletion of sequences outside the ROCK-I/Kinectin homology also reduce RhOA binding in both two-hybrid and overlay assays, although the precise effects vary, presumably owing to the different assay conditions used (5, 43). In agreement with a previous study (5) we found that mutation of sequences highly conserved between ROCK-I and ROCK-II substantially reduced interaction with RhOA in both our assays. These sequences might act to stabilize the interaction between wild type Cdc42 and ROCK-I detected by our assays is specific, because specificity controls with the interaction domains of PAK65, an MSE55-related protein, and PKN clearly demonstrate Cdc42/Rac-specific and RhOA-specific interactions by the CRIB and REM-1 interaction domains. We have also observed interaction between GTPase binding fragments of ROCK-I and activated Cdc42.V12 in microinjection assays in mammalian cells. The discrepancy between our data and those obtained by others using overlay assays may reflect the stringency of the overlay assay compared with the two-hybrid and affinity chromatography approaches used here.

Sequence comparison of the Kinectin cDNA recovered from the two-hybrid assay with the minimal RhOA-binding domain of ROCK-I (5, 43) revealed a 20 amino acid homology between the two proteins. This sequence, which is unrelated to that of the mDia2 RhO interaction domain, is both necessary for interaction with GTP-bound RhOA and Cdc42 and can by itself can interact weakly with these GTPases. Intriguingly, the distinct Kinectin cDNA previously isolated as a potential RhOA effector also contains a sequence related to the ROCK-I/Kinectin homology region (15). The ROCK-I/Kinectin homology occurs within a region of predicted extended coiled-coil structure, and the REM-1 motif that mediates interactions with PKN1/PRK1, Rhotekin, and Rhophilin (14) also may have helical character. Sequences related to the ROCK-I/Kinectin homology are present in heterotrimeric G protein β subunits and in the yeast two-component protein Skn7, also within putative coiled-coil regions. However, although we could demonstrate that both β2 and Skn7 interact with GTP-bound RhOA and Cdc42 and that the interaction is dependent on the ROCK-I/Kinectin homology, the strongly helical character of the sequence has precluded its use as a search string in data base searches for further RhO-interacting proteins.

The ability of the isolated ROCK-I/Kinectin homology to bind RhOA weakly in vitro suggests that it makes direct contacts with the GTPase. However, previous studies of ROCK-I have shown that point mutations or deletion of sequences outside the ROCK-I/Kinectin homology also reduce RhOA binding in both two-hybrid and overlay assays, although the precise effects vary, presumably owing to the different assay conditions used (5, 43). In agreement with a previous study (5) we found that the ability of sequences highly conserved between ROCK-I and ROCK-II substantially reduced interaction with RhOA in both our assays. These sequences might act to stabilize the interaction...
The idea that Skn7 is a Rho1 effector. Consistent with this, a number of reports have also implicated in other protein interactions such as the ROCK-I/Kinectin homology as required for the cell wall (33), as would be expected if Skn7 were titrating Rho1. Level overexpression of Skn7 is lethal owing to weakening of the cell wall, possibly at the level of cell wall functions in the oxidative stress response (44) and in G1 phase (16, 46, 51). It will be necessary to examine the behavior of appropriate point mutants in suitable functional assays to assess the significance of these interactions.

Our results show that yeast Skn7 can interact with both yeast Rho1 and its mammalian homolog RhoA and that the ROCK-I/Kinectin homology is also required for the in vivo interactions are functionally significant. The ROCK-I/Kinectin homology is also required for the function, deletion of this region inactivated the protein.

The significance of the potential interaction of Rho family GTPases with heterotrimeric G protein effector proteins in vivo provides strong support for the idea that Skn7 and the heterotrimeric G protein and the putative exchange factor mNET1 as targets of Skn7 function, deletion of this region inactivated the protein. SKN7 functions in the oxidative stress response (44) and in G1 cyclin synthesis (33), but its mechanism of action is not yet understood. Several observations suggest that it also plays a role in cell morphogenesis, possibly at the level of cell wall synthesis (31–33). In particular, skn7 pck1 double mutants are inviable owing to massive lysis at the small-budded stage of the cell cycle (32, 33), a phenotype highly reminiscent of rho1 mutants (22). These observations suggest that Skn7, like Pkc1, might be a Rho1 effector, and consistent with this notion, high level overexpression of Skn7 is lethal owing to weakening of the cell wall (33), as would be expected if Skn7 were titrating Rho1. Our demonstration that Rho1 and Skn7 can physically interact and that the region of the protein that mediates the interaction is required for Skn7 function in vivo provides strong support for the idea that Skn7 is a Rho1 effector. Consistent with this, a multicopy plasmid expressing Rho1 partly suppresses the lethality induced by Skn7 overexpression.

In summary, we have identified a new Diaphanous-related protein and the putative exchange factor mNET1 as targets of RhoA and Cdc42. A region of similarity between the RhoA-related sequences in yeast Skn7 and the heterotrimeric G protein and the putative exchange factor mNET1 as targets of Rho family GTPases with heterotrimeric G protein interactions in vivo provides strong support for the idea that Skn7 is a Rho1 effector. In summary, we have identified a new Diaphanous-related protein and the putative exchange factor mNET1 as targets of RhoA and Cdc42. A region of similarity between the RhoA-related sequences in yeast Skn7 and the heterotrimeric G protein and the putative exchange factor mNET1 as targets of Rho family GTPases with heterotrimeric G protein interactions in vivo provides strong support for the idea that Skn7 is a Rho1 effector. In summary, we have identified a new Diaphanous-related protein and the putative exchange factor mNET1 as targets of RhoA and Cdc42. A region of similarity between the RhoA-related sequences in yeast Skn7 and the heterotrimeric G protein and the putative exchange factor mNET1 as targets of Rho family GTPases with heterotrimeric G protein interactions in vivo provides strong support for the idea that Skn7 is a Rho1 effector.