

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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Arthur S. Alberts^{‡§}, Nicolas Bouquin[¶], Leland H. Johnston[¶], and Richard Treisman^{‡**}

From the [‡]Transcription Laboratory, Imperial Cancer Research Fund Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX and [¶]Division of Yeast Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom

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 Kinectin, mDia2 (a p140 mDia-related protein), and the guanine nucleotide exchange factor, mNET1. ROCK-I, Kinectin, 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 Kinectin 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/Kinectin sequence homology are present in heterotrimeric G protein β subunits and in the *Saccharomyces cerevisiae* Skn7 protein. We show that β 2 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/Kinectin 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, 11–13), but the functions of the PKN/PRK kinases remain obscure. RhoA also interacts with several apparently non-catalytic effectors including RhoGAP, Rhotekin, Citron, the myosin-binding subunit of myosin light chain phosphatase (MBS),¹ p140 mDia, Kinectin, 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 Rlm1 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 Rho1 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 ISXPX₂ or ₃FXHX₂(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 RhoGAP and has the consensus (L/I/F)X₂(E/K)X₂(V/I/L)X₂GX(E/K/R)(N/R/Q) (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 RhoGAP 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, Kinectin, 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 Kinectin, a p140 mDia-related protein, and the mouse homolog of the NET1 GEF. Comparison of the ROCK-I and Kinectin 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/Kinectin homology region is required for the func-

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§ Supported in part by a postdoctoral fellowship from the Howard Hughes Medical Institute. To whom correspondence should be addressed. Present address: UCSF Cancer Center, Box 0128, San Francisco, CA 94143-0128. Tel.: 415-502-1713; Fax: 415-502-3179; E-mail: alberts@cc.ucsf.edu.

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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 γ S, guanosine 5'-3-O-(thio)triphosphate; 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.

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 U00485; Ref. 31) and human $\beta 2$ subunit (GenBank™ accession number M16538; Ref. 35) coding sequences were obtained by PCR and their DNA sequences confirmed.

GAL4 DNA-binding Domain Fusions—Activated 9E10-tagged RhoA and Cdc42 were subcloned from mammalian expression plasmids (36) as *NcoI* + *Xho* fragments into pGBT9; the RhoA CAAX motif was inactivated by a C190S mutation introduced by PCR and that of Cdc42 by truncation generating Cdc42-(1–178)-ID. Yeast Rho1 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.³ The plasmids encode the following sequences C-terminal to the Gal4 activation domain. cDNA clone D1 encodes IWNSDPREFT-(ROCK-I codons 300–1030)-KKKVNRSRD. cDNA clone D4 encodes IWNSDPNLPSSP-(ROCK-I codons 456–1028)-VNLERSMNRYY. cDNA clone D9 encodes IWNSDPREFT-(ROCK-I codons 349–1025)-G-ELERSMNRYY. GAD-ROCK-(831–1010) encodes IEFPM-(ROCK-I codons 831–1010)-DLQRFMNRYY. GAD-ROCK-(831–1010)/TT is the same as GAD-ROCK-(831–1010) but with K1005T/L1006T. GAD-ROCK-(831–1010)- Δ HR encodes ISGRS-(ROCK-I codons 831–1010)-FQIYES with codons 950–966 deleted. GAD-ROCK.HR encodes ISGRS-(ROCK-I codons 950–972)-FQIYES. GAD-Kinectin-(1053–1327) (cDNA clones D2 and D3) encodes IWNSDPRLDLP-(Kinectin codons 1053–1327). GAD-Kinectin-(1053–1327)- Δ HR encodes IEFPMGRDLP-(Kinectin codons 1053–1327) with codons 1191–1215 replaced by GS. GAD-mDia2-(47–257) (cDNA clone D7) encodes mDia2 IWNSDPREF-(mDia2 codons 47–800)-VNSREIYES. GAD-NET (clone D5) encodes IWNSDPRLDLP-(15 codons from mNET1 5'UT)-(mNET1 1–595). GAD-SKN7 encodes ISGRS-(Skn7 codons 1–623). GAD-SKN7 Δ HR encodes IEF (Skn7 codons 1–623) with codons 237–260 replaced by G. GAD- $\beta 2$.WT encodes ISGRS-(Human $\beta 2$ codons 1–340). GAD- $\beta 2$ Δ HR encodes IEFPM-($\beta 2$ codons 24–340). GAD- $\beta 2$ HR encodes ISGRS-($\beta 2$ codons 1–32)-LEIPDL. GAD-PKN.N encodes IEFPM (PKN codons 1–511). GAD-PAK.N encodes IEFPMAGS-(Rat PAK α codons 1–252)-RRPA-EIYES.

GST Fusion Proteins—All fusion proteins were made using pGEX-KG and encode the following sequences. pGEX-RhoA.WT and pGEX-Cdc42WT encode wild type RhoA and Cdc42, respectively. GST-ROCK-(831–1010) encodes (ROCK-I codons 831–1010)-LELKLNSS. GST-ROCK-(831–1010)/TT is the same as GST-ROCK-(831–1010) with K1005T/L1006T. GST-ROCK-(831–1010)- Δ HR encodes ROCK-(831–1010)-ES, with codons 950–966 deleted. GST-ROCK.HR encodes ROCK-(950–972)-F. GST-Kinectin-(1053–1327) encodes Kinectin codons 1053–1327. GST-Kinectin-(1053–1327)- Δ HR is GST-Kinectin-(1053–1327) with codons 1191–1215 replaced by GS. GST-mDia2-(47–257) encodes (mDia2 codons 47–257)-QLNSS. GST-NET encodes mNET1 (codons 122–595). GST-SKN7 encodes Skn7 1–623 from Lee Johnston. GST-SKN7 Δ HR encodes (Skn7 codons 1–623) with codons 237–260 replaced by G. GST- $\beta 2$.WT encodes ($\beta 2$ codons 1–340). GST- $\beta 2$ Δ HR encodes ($\beta 2$ codons 24–340). GST- $\beta 2$ HR encodes ($\beta 2$ codons 1–32)-LELKLNSS. GST-PAK.N encodes (Rat PAK α codons 1–252)-EIRRLKLNSS. GST-PKN.N encodes (PKN codons 1–511).

Two-hybrid Screen and Yeast Manipulations—Yeast strains and manipulations were as described previously (33, 37, 38). For the two-hybrid screen, yeast strain HF7c carrying pGBT9-RhoA.V14/S190 was used in conjunction with a mouse T-helper cell cDNA library in pSE1107 (39). 4 million transformants were plated onto selective plates lacking histidine and grown for 3 days at 30 °C. Colonies were re-screened for expression of the *lacZ* marker after lifting onto nitrocellulose filters (38).

Recombinant Proteins—Overnight cultures were diluted 1:10 to 50 ml, grown for 3 h, then lysed by sonication in 5 ml of RB (100 mM NaCl, 5 mM MgCl₂, 25 mM Tris, pH 7.2) with protease inhibitors, adsorbed to 0.5 ml of glutathione-Sepharose 4B beads, and washed extensively. RhoA.WT-9E10 and Cdc42.WT-9E10 proteins were released by overnight incubation with thrombin (5 units; Sigma) at 4 °C in RB; throm-

TABLE I
cDNAs recovered in two-hybrid screen

cDNA	Identity ^a	Codons ^b
D1	Mouse ROCK-I (U58512)	300–1030
D4		456–1028
D9		349–1025
D2	Mouse Kinectin (L43326)	1053–1327
D3		
D5	Mouse NET1 Dbl domain bearing Rho family GEF 82% identical to human NET1 (U02081)	1–595
D7	mDia2 (N-terminal EST: AA415402) related to p140 mDia (U96963)	47–800 ^c

^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.

^c 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 mDia sequence. Full details of the mDia2 cDNA clone will be published elsewhere.

bin was removed by adsorption to *p*-aminobenzamidine-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.

In Vitro Binding Assay—Equimolar amounts of each GST-fusion protein (~100–300 ng) were bound to glutathione-Sepharose beads and incubated with 10 ng of GTP γ S- 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 detected by immunoblotting with the 9E10 antibody.

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). Plasmids 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.⁴ Plasmid D7 contains a cDNA related to p140 mDia, as identified in a previous screen for RhoA effector proteins (17) which is related to the *Drosophila* gene *Diaphanous* (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 mDia and with *Drosophila Diaphanous* (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

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

⁴ A. S. Alberts and R. Treisman, manuscript in preparation.

mDia2	1	M E R H R A R A L G R D S K S S R R K G L Q S A P P A G P Y E P G E K R P K L H L N I R T L T D D M L D K	*
p140mDia	1	M E P S G G G L G P G R G T R D K K K G R S P D E L P A T G G D G G K H K K	
Dm Dia	1	M S R H E K T K S T G G L L D S	
mDia2	54	F A S I - - R I P G S K K E R P P L P H L K T V S G I S D S S S L S S E T M E N N P K A L P E S E V L K L	
p140mDia	39	F L E R - - F T S M R I K K E K E K P N - - - S A H R N S S A S Y G D D P T A Q S L Q D I S D E Q V L V L	
Dm Dia	18	L F G R P S K S K G G T I S S G T L A H G G R P V S A D N Y V V P G V E D F E Q Y I Q Q L S V A E L D A K	
mDia2	105	F E K M M E D M N L N E D K K A P L R E K D F G I K K E M V M Q Y I N T A S K T G S L R S S R Q I S P Q E	
p140mDia	87	F E Q M L V D M N L N E E K Q Q P L R E K D I V I K R E M V S Q Y L H T - S K A G M N Q K E S S R S A M M	
Dm Dia	71	F L E I I E D M N I P K D K R E P L L A K S K E E R Q K M I M W H L K G K N S L E R S A N S R F E K P I D	
mDia2	158	F L H E L K M G Y T D E R - L F T Y L E S L R V S L T S H P V S W V Q S F C H E G L G L L D I L E K L I	
p140mDia	139	Y I Q E L R S G L R D M H - L L S C L E S L R V S L N N N P V S W V Q T F G A E G L A S L L D I L K R L H	
Dm Dia	124	V V E Y L Q N G E H S T H K V Y Q C V E S L R V A L T S N P I S W I K E F G V A G T G T I E K L L A R S K	
mDia2	210	N - - G Q I Q E K V V K K T Q H K V I Q C L R A L M N T Q Y G L E R I M S D K R S - - L S L L A K A M D	
p140mDia	191	D E K E E T S G N Y D S R N Q H E I I R C L K A F M N N K F G I K T M L E T E E G - - I L L L V R A M D	
Dm Dia	177	N N A S Y - - - - - E K I E F E A I R C L K A I M N N T W G L N V V L N P D Q H S V V L L L A Q S L D	

FIG. 1. **Sequence of the RhoA-binding domain of mDia2.** The sequence is shown compared with the appropriate regions of the p140 mDia (17) and *Drosophila* Diaphanous (41) proteins. The asterisk marks the start of the mDia2 sequences isolated in clone D7. Identical residues in two or more of the sequences are reverse-shaded, and similar residues are highlighted.

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 effectors⁵; 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 γ 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 Kinectin, ROCK-I, and mDia2 all represent potential effectors for both RhoA and Cdc42; we show elsewhere that mNET1 is a RhoA GEF⁴ (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

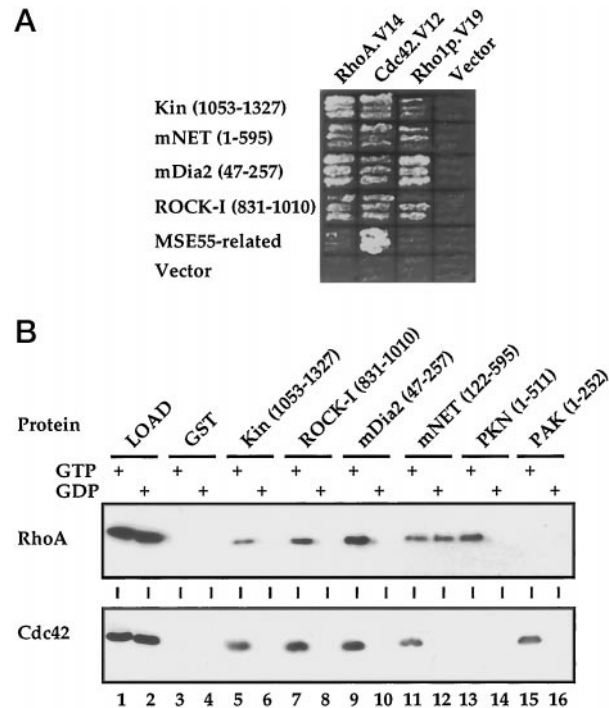


FIG. 2. **Interactions of effector proteins with Rho family GTPases.** A, yeast two-hybrid assay. HF7C yeast were transformed with combinations of expression plasmids expressing GAL4-DNA-binding domain/GTPase or Gal4 activation domain/effector protein fusion genes. Three independent transformants in each case were assayed for growth after 3 days at 30 °C on selective medium lacking histidine and containing 2 mM aminotriazole. Interaction with RhoA.V14, Cdc42.V12, and Rho1.V19 was tested with each cDNA. The MSE55-related cDNA⁵ encodes a CRIB-domain protein (34). B, *in vitro* protein interaction assays. 9E10-tagged RhoA (top panel) or Cdc42 (bottom panel) were preloaded with either GTP γ S or GDP as indicated. Each GTPase (10 ng) was incubated with glutathione beads carrying equimolar amounts of the indicated GST-effector fusion proteins (100–300 ng). Following washing, bound proteins were eluted and fractionated by SDS-PAGE. GTPases were detected by immunoblotting using the 9E10 antibody.

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-

⁵ A. S. Alberts, unpublished data.

FIG. 3. Homologies between ROCK-I and Kinectin. A, comparison of the ROCK RhoA-binding regions with Kinectin cDNA clone D2. Vertical lines indicate identity and asterisks similarity. The extents of the minimal ROCK interaction domains are defined previously in human ROCK-I (43) and rat ROK- α (5) are indicated by underlining. The ROCK-I/Kinectin homology region is overlined. The deletions investigated in this paper are indicated by large reverse-shaded boxes and the location of the KL \rightarrow TT double point mutation by a small reverse-shaded box. B, comparison of the ROCK-I/Kinectin homology region with sequences in ROCK-II, β 2, Skn7, and Kinectin (N-terminal sequence). Similarity between four or more of the sequences is shown by shading and identity by reverse shading.

A			
mKinectin	1177	SFTASERELERLRQEN...KQMDNRREHHPHEMLEKKAEMERSTYVMEVRELKDLLTELQ	
mROCK-I	930	EITDKDHTVSRLEETNSVLTKQTEMERKENEENRMRTAEEYKLLKKEEIN...NLKAAFE	
mROCK-II	950	ELTEKDTTASLEETNRTLTSQVANLANEKEELNNKLKDSQEQLSKLKDEMSAAAIAKQFE	
mKinectin	1135	KKLDDSYSEAVRQNEELNLLKTQLNETHS.KLQNEQTERKKVADDLHKAQQSLNSIHSKISL	
mROCK-I	990	KNISTERTLTKQAVN...PAETIMNRKDFKIDRKANTQDLRKKEKENRKL.QLELNQEREKFNQ	
mROCK-II	1012	KOLLNERTLTKQAVNKLAEIMNRKE...PVKRGSDTDVRRKEKENRKL.HMELKSEREKLTO	
B			
mKinectin	1192	N K D M E N L R R E R E H L E M E L E K A E M E	1115
mROCK-I	949	T K D I E M L R K E N E E L N E R M R T A E E H	972
mROCK-II	893	T S D V A N L A N E K E E L N N K L K D S Q E Q	916
β 2	1	M S E L E Q L R Q E A E Q L R N Q L R D A R K A	23
Skn7	237	K D A F G N L R R R V D K L Q K E L D M S K M E	260
mKinectin	831	E K T Q V A L K Q E I E V L K E E I G N A Q L E	854

imum homology between the ROCK proteins corresponds to ROCK-I residues 995–1014 (5, 7) a region of substantial similarity between ROCK-I and Kinectin is found N-terminal to this, corresponding to ROCK-I residues 950–972 (Fig. 3A). This region lies within the “leucine zipper” region of ROCK-I and is also within a coiled-coil region in Kinectin. No substantial sequence homology was found with mDia2 (data not shown). We used the homology between ROCK-I and Kinectin to screen the sequence data bases for similar sequences using the Blastp program (NCBI). Among many coiled-coil proteins identified, this search detected a second Kinectin sequence element N-terminal to that in our cDNA clone (residues 832–854) and a region at the N terminus of heterotrimeric G protein β subunits. We also observed that the sequence of the *S. cerevisiae* SKN7 gene, which interacts genetically with the *RHO1-PKC1* pathway (31–33), also contains a region of similarity to the ROCK-I/Kinectin homology, again within a region predicted to form a coiled-coil structure (Fig. 3B; see “Discussion”). These sequences are compared in Fig. 3B.

The ROCK-I/Kinectin Homology Is Required for Interaction with Rho Proteins—To investigate the significance of the ROCK-I/Kinectin homology, 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, rows 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-(831–1010) generates a protein that does not interact with RhoA or Cdc42 in either assay (ROCK-I-(831–1010)- Δ HR: Fig. 4A, row 3; Fig. 5A, lanes 6). Similarly deletion of the homology region from Kinectin also abolished interaction with RhoA and Cdc42 in both assays (Kinectin-(1053–1327)- Δ HR: Fig. 4A, row 7; Fig. 4B, row 2; Fig. 5A, lanes 3 and 4). The ROCK-I/Kinectin homology region is therefore required for interaction of both proteins with RhoA and Cdc42.

We also examined whether Skn7 and heterotrimeric G protein β subunit, which contain sequences related to the ROCK-I/Kinectin homology, also interact with Rho family GTPases. Sequences encoding β 2 and Skn7 were amplified by PCR and inserted into appropriate plasmids for use in the two-hybrid and *in vitro* interaction assays. In addition, derivatives of each protein were constructed in which the ROCK-I/Kinectin homology region was deleted (Skn7 Δ HR and β 2 Δ HR). In the two-hybrid assay, both proteins interacted with RhoA.V14, yeast

Rho1.V19, and Cdc42.V12; deletion of the homology region from either protein reduced interactions with all GTPases to background levels (Fig. 4A, rows 8–11; Fig. 4B rows 3–6). Both β 2 and Skn7 also bound wild type RhoA.GTP and Cdc42.GTP in the *in vitro* 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 β 2 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 β 2 (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 β 2 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-I/Kinectin 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 β 2-(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 is an essential structure mediating 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

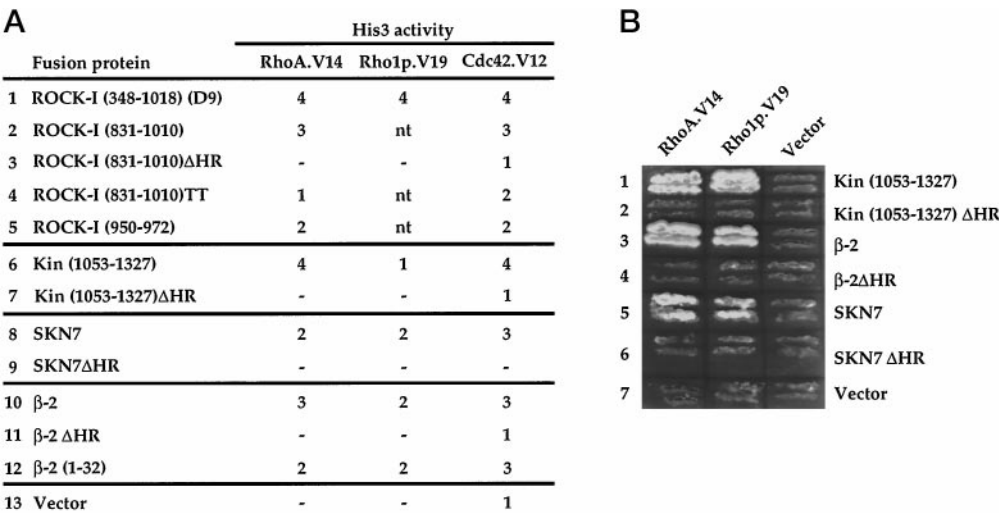


FIG. 4. **The ROCK-I/Kinectin homology is necessary and sufficient for two-hybrid interactions with RhoA.V14, Rho1.V19, and Cdc42.V12.** A, analysis of different effector protein mutants. Proteins lacking the ROCK-I/Kinectin homology region are denoted ΔHR. Interaction strength was assessed by growth 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 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.

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 *swi4^{ts} swi6Δ* 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.⁶ 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 *swi4^{ts} 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

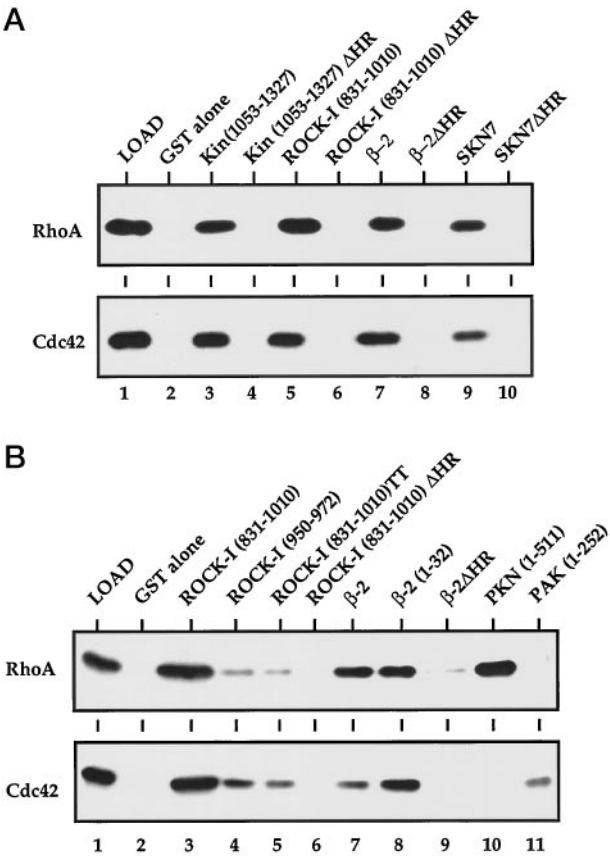


FIG. 5. A, the ROCK-I/Kinectin homology is necessary for *in vitro* interactions with RhoA.V14 and Cdc42.V12. 9E10-tagged wild type RhoA (top panel) or Cdc42 (bottom panel) were preloaded with either GTPγS or GDP. Each GTPase (10 ng) was incubated with glutathione beads carrying equimolar amounts of 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.

⁶ N. Bouquin and L. H. Johnston, unpublished data.
⁷ N. Bouquin and L. H. Johnston, manuscript in preparation.
⁸ N. Bouquin, unpublished data.

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

Gene ^a	pGAL overexpression ^b	<i>swi6Δ</i> p3MCB- <i>lacZ</i> ^c	<i>swi4^{ts}</i> <i>swi6Δ</i> ^d	RhoA ^e	<i>cdc42</i> ^f	H ₂ O ₂ ^g	<i>pkc1-8</i> <i>skn7Δ</i> ^h	<i>swi4^{ts}</i> <i>swi6Δ</i> <i>skn7Δ</i> Yep MBP1 ⁱ
1. <i>SKN7</i>	Lethal	+++	+	+	—	+	+	+
2. <i>skn7Δ427N</i>	Lethal	+++	—	ND	—	+	—	+
3. <i>skn7ΔHR</i>	Viable	+/-	—	—	—	—	—	—
4. <i>skn7Δ353-623</i>	ND ^j	—	—	—	+	—	—	ND

^a *SKN7*, intact wild type gene; *Skn7Δ427N*, mutation of the aspartate phosphoacceptor in the receiver domain (32, 33); *Skn7ΔHR*, deletion of the ROCK-I/Kinectin homology region (residues 237–260); *Skn7Δ*-(353–623), deletion of the C-terminal deletion of *SKN7*.

^b High overexpression of *SKN7* from the *GAL* promoter (33).

^c 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.

^d Suppression of the *swi4^{ts}* *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.

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

^f 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.

^g *skn7Δ* cells are acutely sensitive to H₂O₂ (44). +, suppression of sensitivity.

^h *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 (+).

ⁱ *swi4^{ts}* *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.⁷ High copy *SKN7* derivatives were scored for their ability to permit growth of a *skn7 swi4^{ts} swi6Δ* strain at 37 °C. +, growth at 37 °C.

^j 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 Rho1 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 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 Rho family guanine nucleotide exchange factor activity (for review see Ref. 45); indeed, mNET1 acts as a RhoA GEF both *in vitro* and *in vivo*.⁴ In addition to significant sequence homology between mDia2 and p140 mDia within the RhoA-binding domain, mDia2 also contains a region homologous to the p140 mDia formin homology domain; characterization of the mDia2 protein is in progress.

Our data implicate ROCK-I, Kinectin, and mDia2 as effectors for both RhoA and Cdc42. However, although previous studies of ROCK-I using overlay and two-hybrid assays broadly concur concerning its interactions with RhoA, its ability to interact with other Rho family proteins has been contentious. Two studies reported weak interaction between ROCK-I and activated Cdc42, although one observed no interaction with wild type Cdc42 (46, 47); interactions with activated Rac1 have also been reported (47, 48). We are confident that 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.⁵ 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 PKN/PRK1, Rhotekin, and Rhophilin (14) also may have helical character.⁴ 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/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.⁹

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 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

⁹ J. Sgouros, unpublished observations.

secondary structure of the ROCK-I/Kinectin homology; alternatively, they might represent a second GTPase docking site. Further studies will be necessary to resolve this issue.

The significance of the potential interaction of Rho family GTPases with heterotrimeric G protein β subunits remains unclear. Our results are in agreement with a previous report that indicated that G $\beta\gamma$ can bind both RhoA and Rac1 and may be involved in membrane targeting of these proteins (49). The putative Rho interaction surface at the β subunit N terminus has also been implicated in other protein interactions such as binding of the Ste20 kinase and Cdc24 GEF in *S. cerevisiae* (50, 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 region is required for this interaction. The ROCK-I/Kinectin homology is also required for the biological activity of Skn7 *in vivo*; in a number of assays for 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 pkc1* 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.⁸

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-binding domains of ROCK-I and Kinectin was used to identify related sequences in yeast Skn7 and the heterotrimeric G protein subunit $\beta 2$. These proteins were also shown to interact with RhoA and Cdc42, and studies of Skn7 indicate that these interactions are functionally significant *in vivo*. Future studies will be directed toward the detailed characterization of these interactions.

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