The Rho GTPase, Cdc42, regulates a wide variety of cellular activities including actin polymerization, focal complex assembly, and kinase signaling. We have identified a new family of very small Cdc42-binding proteins, designated SPECs (for Small Protein Effector of Cdc42), that modulates these regulatory activities. The two human members, SPEC1 and SPEC2, encode proteins of 79 and 84 amino acids, respectively. Both contain a conserved N-terminal region and a centrally located CRIB (Cdc42/Rac Interactive Binding) domain. Using a yeast two-hybrid system, we found that both SPECs interact strongly with Cdc42, weakly with Rac1, and not at all with RhoA. Transfection analysis revealed that SPEC1 inhibited Cdc42-induced c-Jun N-terminal kinase (JNK) activation in COS1 cells in a manner that required an intact CRIB domain. Immunofluorescence experiments in NIH-3T3 fibroblasts demonstrated that both SPEC1 and SPEC2 showed a cortical localization and induced the formation of cell surface membrane blebs, which was not dependent on Cdc42 activity. Cotransfection experiments demonstrated that SPEC1 altered Cdc42-induced cell shape changes both in COS1 cells and in NIH-3T3 fibroblasts and that this alteration required an intact CRIB domain. These results suggest that SPECs act as novel scaffold molecules to coordinate and/or mediate Cdc42 signaling activities.

The ability of Cdc42 to influence diverse activities stems from its interactions with a large number of different kinase and non-kinase effector proteins. Although GTP-bound Cdc42 usually interacts with downstream effector proteins containing the conserved binding motif called a CRIB domain (7), some downstream Cdc42 effector proteins such as IQGAP do not contain CRIB domains (8, 9). To date, six distinct families of CRIB domain-containing Cdc42 effector proteins have been identified: PAK (10, 11), MRCK (12), ACK (13), MLK (7, 14), WASP (15, 16) and M555/CEP/BOB family, which is the most structurally diverse. This family, consisting of five members, all inducers along Cdc42-containing cellular extensions in NIH-3T3 fibroblasts (17, 18).

With so many different Cdc42 effector proteins, many of which may coexist in a single cell, competition and/or some mechanism for coordination must exist to ensure that the proper Cdc42 signal is propagated. Although many individual Cdc42 effector proteins have been studied, little is known about how these effector proteins cooperate and/or compete with each other, either in regulating the cytoskeleton or in kinase signaling. Here, we have identified a novel family of Cdc42 effector proteins that may play a role in this higher level of coordination. This new family, designated SPEC (for Small Protein Effector of Cdc42) has two human members, SPEC1 and SPEC2. Both are very small and are highly conserved. SPECs appear to have multifaceted activities, of which some are independent of Cdc42 binding and some are dependent on Cdc42

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The nucleotide sequence(s) reported in this paper have been submitted to the GenBank®/EBI Data Bank with accession number(s) AF187845 and AF189692.*

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†The abbreviations used are: CRIB, Cdc42/Rac interactive binding domain; AKAP, A-kinase anchoring protein; EST, expressed sequence tag; JNK, c-Jun N terminal kinase; SPEC, small protein effector of Cdc42; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; contig, group of overlapping clones; HA, hemagglutinin.
binding. For instance, expression of SPECs in NIH-3T3 fibroblasts induced membrane blebbing. SPEC-induced blebbing did not require Cdc42 activity because blebbing still occurred with a CRIB domain mutant of SPEC or in the presence of the dominant negative Cdc42. SPEC1 blocked both Cdc42-induced JNK activity and altered Cdc42-induced morphological changes in COS1 cells and in NIH-3T3 fibroblasts in a manner that required an intact CRIB domain and, thus, was dependent on Cdc42 binding. Together these results suggest that SPECs act as novel scaffold molecules to coordinate and/or mediate Cdc42 signaling activities.

**EXPERIMENTAL PROCEDURES**

**Identification and Cloning of SPECs—**Clones of SPEC1 were identified by a TBLASTN search of the expressed sequence tag (EST) data base at the National Center for Biotechnology Information (NCBI) using the 16-amino acid CRIB core sequence of MEE55 (7) (ISHPLGDFRTTHMHVGR) as a query. Several of these human EST clones were obtained from the I.M.A.G.E. consortium (clone ID numbers: 257442, 139233, 160147) and sequenced on an Applied Biosystem 377 DNA sequencer. The GenBank™ accession number of SPEC1 is AF187845. Furthermore, EST clones for human and mouse SPEC1 are quite abundant and are represented by the NCBI Unigene identifiers Hs.22065 and Mm.28189, respectively.

Additional TBLASTN searches of the non-redundant nucleotide data base using the amino acid sequence of SPEC1 as a query identified three well separated DNA sequences that if transcribed as a single mRNA and properly spliced might encode a second SPEC-like protein. Two adapter-primers, 5’-CAGGGATCCGATGGATTCTTGGTGTGTTG-3’ and 5’-GAGCTCGAACGCCTCGCTCTGACCCG-3’, derived from these genomic sequences and corresponding to each end of the putative coding sequence, were synthesized. These two primers were used in reverse transcription-PCR with MCF-7 breast cancer cell RNA as template. After PCR, an approximately 250-base pair PCR fragment was obtained, cut with BamHI/HindIII, and subcloned into the BamHI-XhoI site of pCASP2, a mammalian expression vector (17). DNA sequencing revealed that the nucleotide sequence of this PCR product was, as expected, derived from the three different 5’-del1 genomic fragments and encodes a protein, designated SPEC2, that was quite similar to SPEC1. SPEC2 has GenBank™ accession number AF189692.

**Yeast Two-hybrid Assays—**The yeast two-hybrid assay was performed in the Y190 yeast strain using the pYTH GAL4 DNA binding domain yeast vectors and pACT-II GAL4 activation domain yeast vectors formed in the Y190 yeast strain using the pYTH GAL4 DNA binding domain yeast vector and pACT-II GAL4 activation domain yeast vectors (30). cDNAs for SPEC1 (amino acids 2–79) or SPEC2 (amino acids 2–84) were subcloned downstream of the GAL4 DNA binding domain in pYTH-9, and integrated strains were generated as described (30). Deletion mutants of SPEC1 consisting of amino acids 2–27 (SPEC1-del1) or amino acids 48–79 (SPEC1-del2) were constructed in the same way. Wild type and activated mutants of Cdc42, Rac, and Rho were subcloned downstream of the GAL4 activation domain in pACT-II. Protein-protein interactions were detected by assaying for β-galactosidase activity in a filter assay or by growth on nutrient agar plates lacking histidine (30).

**Mammalian Expression Vectors for SPEC1, SPEC2, and Mutants—** The coding sequence of SPEC1 was amplified by PCR from the I.M.A.G.E. cDNA clone (ID 22978) using the two primers 5’-CTAGAATTG-3 and the QuickChange mutagenesis kit (Stratagene). Four single or amino acids 48–79 (SPEC1-del1) were constructed in the same way. Wild type and activated mutants of Cdc42, Rac, and Rho were subcloned downstream of the GAL4 activation domain in pACT-II. Protein-protein interactions were detected by assaying for β-galactosidase activity in a filter assay or by growth on nutrient agar plates lacking histidine (30).

**RESULTS**

**Identification of Genes for SPEC1 and SPEC2—**Most known Cdc42-inducing proteins contain a conserved core domain, the CRIB domain (7). We looked for additional proteins that might bind Cdc42 by searching the human EST data base for sequences similar to the CRIB domain of the non-kinase Cdc42 effector protein MEE55/CEP1 (17) and identified many cDNAs encoding the same small protein. The DNA sequences of several independent human clones comprising a contig spanning 1.8 kilobases each showed an open reading frame of 79 amino acid residues, which we designated SPEC1 (Fig. 1). These cDNAs encoding the SPEC1 protein contained a Kozak consensus sequence and an upstream in-frame stop codon and did not encode any proteins longer than SPEC1. A second human SPEC family member, designated SPEC2, was identified by searching the non-redundant GenBank™ data base. This search identified three short separated genomic regions from chromosome 5q31, spanning at least 28 kilobases (GenBank™ accession numbers AC001489 and AC001223) that if transcribed and properly spliced would encode a second SPEC-like protein. To clone the SPEC2 cDNA sequence, primers were designed for the two extreme ends of the genomic regions and used in reverse transcription-PCR. This approach yielded a 250-base pair PCR fragment containing an open reading frame of 84 amino acids (Fig. 1). Comparison of this sequence with 5q31 genomic sequences confirmed that the full-length SPEC2 protein was encoded in three exons spanning at least 28 kilobases.

In addition to the human SPEC1 and SPEC2 proteins, we have also identified both SPEC1 and SPEC2 homologs in other organisms. We have identified SPEC1 homologs from mouse (GenBank™ AI472516) and chicken (GenBank™ AI981286) that are 96 and 83% identical to SPEC1 at the amino acid level, respectively (Fig. 1). We have also identified SPEC2 homologs from mouse (GenBank™ AW016119), rat (GenBank™ AA944400), Drosophila (AA207336), and ascidian (Halocynthia roretzi; GenBank™ AV382466) (Fig. 1). Identification of the SPEC proteins in such diverse organisms suggests their function may be conserved through evolution.

SPECs Represent a Novel Family of Cdc42-binding Proteins—SPEC1 and SPEC2 represent two members of a new protein family that are 76% similar over their entire length (Fig. 1) and encode proteins with predicted molecular masses of 7.9 kDa and 8.4 kDa, respectively. Both SPECs contain a
highly conserved N-terminal region and a typical CRIB domain. The CRIB domains of the SPECs extend beyond the CRIB core sequence and contain the consensus sequence DR(S/T)M, where A represents an aliphatic amino acid, and bold letters identify the CRIB core (Fig. 1). In the C terminus of both proteins there is a relatively small conserved sequence containing the nine-amino acid consensus sequence, (V/I)Q(E/N)QM(R/Q)SKG (Fig. 1). This region may be part of an extended high affinity Cdc42 binding site (32, 33).

CRIB-dependent Cdc42 Binding by SPECs—Since proteins containing a consensus CRIB domain will bind Cdc42 and/or Rac (7), we predicted that both SPEC1 and SPEC2 also would interact with Cdc42 and/or Rac. We tested this prediction in a yeast two-hybrid system. Both SPEC1 and SPEC2 interacted strongly with a constitutively activated mutant of Cdc42 (Cdc42-Q61L), weakly with an activated mutant of Rac1 (Rac1-Q61L), and not at all with an activated mutant of RhoA (RhoA-Q63L) between both β-galactosidase filter assay (Fig. 2) and growth on selective media (data not shown). Although interaction of SPECs with wild type Cdc42 and wild type Rac1 was not observed, our results are consistent with yeast two-hybrid experiments using other CRIB-containing Cdc42 effector proteins, such as Wasp (30). Alternatively, SPEC CRIB deletion mutants retaining either the N terminus (SPEC1-del1; amino acids 2–27) or the C terminus (SPEC1-del2; amino acids 48–79) were unable to bind to an activated Cdc42 mutant (Fig. 2). Collectively, these results demonstrate that SPECs can interact with Cdc42 in a CRIB-dependent fashion and suggest that SPECs may function normally in Cdc42-dependent signaling.

SPEC1 Expression Inhibits Cdc42-induced JNK Activity—Since a variety of studies have shown both that Cdc42 (O1, 34) and some Cdc42 effector proteins (14, 20–22, 26) can activate JNK activity, we tested SPEC1 and several SPEC1 mutants for their effect on Cdc42-induced JNK activation. First, an expression construct of human SPEC1 carrying an N-terminal FLAG epitope tag was transfected into NIH-3T3 fibroblasts, and its expression was analyzed by Western blotting using a monoclonal antibody against the N-terminal FLAG epitope tag. Using this approach, SPEC1 was detected in lysates as an ~8 kDa species (data not shown), of which about 1 kilobase is contributed by the epitope tag. Second, several SPEC1 mutants were constructed, including two CRIB mutants and a C-terminal double mutant. The two CRIB mutants, SPEC1-H38A and SPEC1-P33A,H38A, contain alanine substitutions within critical contact sites known to be involved in Cdc42 binding (33, 34). The third mutant, SPEC1-Q62A,K66A, contained mutations within the nine-amino acid region conserved between both SPEC proteins that might be part of an extended high affinity Cdc42 binding site. COS1 cells were cotransfected with the expression vectors for GFP (control) or Cdc42-Q61L, hemagglutinin (HA) epitope-tagged JNK, and FLAG-tagged SPEC1 constructs. After 24 h, transiently expressed HA-JNK was isolated by immunoprecipitation and used for in vitro kinase assays. All SPEC1 constructs tested were not able to stimulate JNK activity on their own (Fig. 3A). Expression of Cdc42 with HA-JNK stimulated kinase activity (Fig. 3, A and B). Cotransfection of cells with wild-type SPEC1 significantly reduced the Cdc42-induced JNK activation (Fig. 3, A and B). In contrast, SPEC1-P33A,H38A, H41A or SPEC1-Q62A,K66A were markedly less effective at blocking Cdc42-induced JNK activation (Fig. 3, A and B). In addition, a similar failure to block Cdc42-induced JNK activation was also observed with the single CRIB domain mutant (data not shown). Although we cannot rule out the possibility that our overexpression studies have resulted in abnormally high levels of SPEC1, which may nonspecifically inhibit Cdc42 signaling pathways, these results may also suggest that SPEC1 modulates JNK activity by binding or sequestering Cdc42 through a CRIB-dependent interaction.

SPEC1 Expression Inhibits Cdc42-induced Morphological Changes in COS1 Cells—We next examined the cellular distribution of epitope-tagged SPEC1 expression by immunofluorescence. In COS1 cells, SPEC1 showed diffuse cytoplasmic localization (Fig. 4B). Additionally, SPEC-expressing cells did not show altered actin structures or cell morphology (data not shown). We next determined whether SPEC1 influenced Cdc42 function when co-expressed with Cdc42. COS1 cells expressing the constitutively active Cdc42-Q61L mutant exhibited a widely spread and flattened phenotype with some filopodia (Fig. 4, A and B). Cotransfection of SPEC1 with Cdc42-Q61L resulted in cells that were more elevated and much less spread than cells expressing Cdc42-Q61L alone (compare Fig. 4, C and D with A) or untransfected cells (data not shown). In contrast to what was seen with wild-type SPEC1, cells expressing Cdc42-Q61L and the CRIB domain mutant (SPEC1-H38A) resembled cells transfected with Cdc42-Q61L alone (compare Fig. 4, E and F with A). These results suggest that SPEC1 modifies Cdc42 function. In these experiments, SPEC1 appears to alter Cdc42 activity by binding to it via the CRIB domain. These results suggest that SPECs may function to block the interaction of Cdc42 with other effector proteins, although we cannot
rule out the possibility that the observed blocking activity was due to overexpression of SPEC1 protein.

SPEC Expression Induces Non-apoptotic Blebbing in NIH-3T3 Fibroblasts—Since SPEC1 expression did not noticeably alter the morphology of COS1 cells, we studied the effects of SPEC1 expression in NIH-3T3 fibroblasts. In NIH-3T3 fibroblasts, SPEC1 displayed a predominant cortical localization (Fig. 5A), and frequently, these transfected cells showed extensive membrane blebbing (Fig. 5A). F-actin stained strongly within the peripheral areas of the blebs but not within the blebs (Fig. 5B). Expression of SPEC2 also showed the same cortical localization, membrane blebbing, and F-actin staining phenotype (Fig. 5, C and D). A similar pattern of cortical staining and blebbing were observed with a myc epitope tag located either at the N or C terminus of SPEC1 and using a 20-fold range of plasmid concentrations (100 ng to 2 μg; data not shown). Although this SPEC-induced membrane blebbing is morphologically similar to the membrane blebbing associated with apoptosis, there is no functional association of the SPEC-induced blebbing with apoptosis. That is, neither nuclear condensation nor annexin-V positive staining, a marker for phosphotidylserine flipping in the membrane, was observed in these transfected cells (data not shown).

Quantitatively, membrane blebbing was observed in 40–60% of the FLAG epitope-tagged SPEC1 transfected cells but only in about 5% of cells expressing the vector-alone control (Fig. 6). We also used a bicistronic expression vector expressing both SPEC1 and EGFP from the same vector to rule out the
possibility that the epitope tags might influence SPEC function. About 40% of the cells expressing the bicistronic SPEC1 construct showed a blebbing phenotype, whereas only 10% were blebbing with the EGFP-alone vector (Fig. 6). Taken together, these results demonstrate that expression of SPECs, whether epitope-tagged or untagged, leads to membrane blebbing in NIH-3T3 fibroblasts.

SPEC1-induced Blebbing Is Independent of Cdc42 Activity—To determine whether any of the three conserved regions in SPECs (see Fig. 1) are necessary for SPEC1-induced membrane blebbing, we examined the phenotype of cells transfected with various SPEC1 mutants. An additional N-terminal mutant, SPEC1-C10A,C11A, was created within two conserved cysteine residues because of the potential role of these residues in lipid modification or protein interactions. Using cell counting it was found that both the positive and negative controls gave the expected results: approximately 44% of N-terminal FLAG-tagged SPEC1-transfected cells blebbed, as compared with only 4% using a vector-alone control (Fig. 7). The C-terminal double point mutant, SPEC1-Q62A,K66A, had no effect on the level of blebbing (Fig. 7). In contrast, the cells expressing the N-terminal mutant (SPEC1-C10A,C11A), which showed a similar level of expression and cortical localization, produced the blebbing phenotype in only 20% of the transfected cells (Fig. 7). Single or triple amino acid substitutions within the CRIB domain of SPEC1 resulted in somewhat fewer blebs, although they still produced significantly more than the negative controls (30% versus 4%; Fig. 7). Additional studies expressing a dominant negative mutant of Cdc42 (Cdc42-T17N) alone did not induce membrane blebbing, and co-expression with SPEC1 did not block membrane blebbing (data not shown). These results support a model whereby SPEC1-induced blebbing does not occur through classical Cdc42-effector interactions and suggest that SPEC1 may act independently of Cdc42 or perhaps upstream of Cdc42 to induce membrane blebbing. These data also confirm that SPEC-induced membrane changes are not directly due to sequestration of Cdc42.

SPEC1 Expression Alters Cdc42-induced Changes in Cellular Morphology in NIH-3T3 Fibroblasts—To more clearly define the relationship between Cdc42 activity and SPECs, we tested the effect of SPEC1/Cdc42 co-expression in NIH-3T3 fibroblasts. In these fibroblasts, expression of Cdc42L61 resulted in cells that predominantly exhibited a membrane ruffling phenotype, possibly through activation of Rac signaling. We then cotransfected SPEC1 or the SPEC1-CRIB mutants (SPEC1-H38A or SPEC1-P33A,H38A,H41A) with constitutively active Cdc42 and quantified by cell counting the number of transfected cells showing a ruffling phenotype. Expression of a constitutively active Cdc42 mutant (Cdc42-Q61L), but not wild type Cdc42 (data not shown), in NIH-3T3 fibroblasts induced marked membrane ruffling in 52% of the transfected cells (Fig. 8, A and B). Co-transfection of SPEC1 blocked ruffling in all but 5% of the transfected cells and increased the number of blebbing cells (Fig. 8, C and D). Coexpression of the SPEC1-H38A mutant resulted in 34% of the cells showing a membrane ruffling phenotype (Fig. 8, E and F) and resembled cells transfected with Cdc42 alone (compare Fig. 8, A and B, with E and F). Similar results were also obtained with the
SPEC1-P33A,H38A,H41A CRIB mutant (data not shown). It is also worthy to note that in these cotransfections experiments, SPEC1 and Cdc42 proteins appear to localize to similar regions within the cells, suggesting that SPECs and Cdc42 may be contained within the same signaling complexes (Fig. 8). As with COS1 cells, these transfections demonstrate that SPEC1 expression led to an altered Cdc42-induced morphology and that this alteration is dependent on the presence of an intact CRIB domain.

**DISCUSSION**

Here we identify a new family of proteins capable of binding to Cdc42, designated SPECs, found in many eukaryotic species. The two human members, SPEC1 and SPEC2, are the smallest known GTPase-binding proteins. Their small size may explain why they were not detected in previous biochemical screens based on binding to Cdc42. Overexpression of different combinations of SPECs, SPEC mutants, and Cdc42 showed that SPEC expression inhibited Cdc42-induced JNK activity. SPEC overexpression also altered or reversed the cellular morphologies produced when Cdc42 is overexpressed in COS1 cells and in NIH-3T3 fibroblasts. The membrane blebbing induced by SPEC overexpression in NIH-3T3 fibroblasts was not observed in COS1 cells, possibly due to quantitative differences in expression levels of SPEC proteins between the two cell types. Nevertheless, these results show that SPECs are capable of modifying Cdc42-dependent signaling at both the biochemical and cellular levels in a CRIB-dependent manner. SPEC binding could prevent the interaction of Cdc42 with other effector proteins. Consistent with this model, a polypeptide containing just the CRIB domain of PAK can effectively inhibit Cdc42 activation of JNK kinase (34) and block transcriptional activation (35), whereas a polypeptide containing the CRIB domain of ACK-1 can act as a Cdc42-specific inhibitor, blocking v-Ha-Ras-induced transformation (36).

However, we do not know if the specific biochemical and biological effects observed here with overexpressed SPECs reflect the normal function of these small proteins. In particular, SPEC overexpression induced membrane blebbing in NIH-3T3 fibroblast that was not blocked by dominant negative Cdc42 expression. Despite these findings, it is still possible that SPECs function in Cdc42-induced morphological changes, since a dominant negative approach may not rescue the abnormal morphology of overexpressed SPEC protein. Furthermore, various studies have shown that non-apoptotic membrane blebs function normally in cell spreading (37, 38) and locomotion (39–41). Mechanistically, membrane blebs occur at sites where the cortical actin is locally depolymerized or detached from the membrane (38, 40, 41) via alteration in cortical actin-binding proteins (40), myosin light chain kinase activity (42, 43), and/or focal complex assembly (43). Thus, it is tempting to speculate that SPECs may function as classical Cdc42 effector proteins by altering the normal signaling pathways leading to actin, myosin, and/or focal complex assembly.

The existence of small proteins that bind important signaling molecules is not unique to Cdc42. Recently, an 18-kDa protein, A-kinase anchoring protein-18 (AKAP18), was found to function as a scaffold protein, coupling protein kinase A signaling to calcium and sodium channels (44–46). Interestingly, AKAP18 and SPECs share many structural and functional similarities. First, both are small proteins: AKAP18, SPEC1, and SPEC2 are 81, 79, and 84 amino acids long, respectively. Second, both bind their ligands, protein kinase A or Cdc42, through their central binding regions. Third, both localize to the plasma membranblebbing.
membrane. Although the membrane localization of AKAP18 involves lipid modification of the N terminus, we have not yet identified the region required to target SPECs to the membrane in NIH-3T3 fibroblasts. Based on these similarities, we speculate that SPECs, like AKAP18, may function as scaffolding molecules to recruit other signaling proteins to Cdc42 complexes. Future studies are aimed at identifying such SPEC-binding partners.

REFERENCES

1. Kroschewski R, Hall A, and Mellman, I. (1999) Nat. Cell Biol. 1, 8–13
2. Drechsel, D. N., Hyman, A. A, Hall, A, and Glotzer, M. (1997) Curr. Biol. 7, 12–23
3. Johnson, D. I. (1999) J. Biol. Chem. 274, 27225–27228