Cdc42 is a Ras-related GTP-binding protein that has been implicated in the regulation of the actin cytoskeleton and cell morphology. In this study, we have identified a protein with a molecular mass of 180 kDa from rabbit liver cytosol (designated p180), which binds preferentially to the GTP- and guanosine 5'-O-(thio)triphosphate-bound forms of Cdc42. Binding of p180 to GTP-bound Cdc42 maintains it in the GTP-bound state. Another cytosolic protein, with an apparent molecular mass of 175 kDa (p175), was also found to interact with Cdc42, but this association showed less dependence on guanine nucleotides. Both p180 and p175 were capable of binding to Rac1 but not to RhoA or Ha-Ras. The limit functional domain of the Cdc42-GAP protein did not compete with p180 or p175 for binding to Cdc42. However, the Cdc42-binding domain from mPAK-3, a member of the PAK (p21 activated kinase) family of serine/threonine kinases, competed with both proteins. The binding of p180 and p175 was inhibited by mutations of the putative effector loop of Cdc42. p180 and p175 also bound less effectively to a Cdc42/Ras chimera in which loop 8 from Ras was substituted for the predicted loop 8 in Cdc42 that includes a 13-amino acid insert present in all Rho family members but absent in Ras. Microsequencing of a p180 peptide revealed 92% identity with the human IQGAP1 protein, while two peptides derived from p175 were 89 and 100% identical to human IQGAP2. These findings identify IQGAP1 and IQGAP2 as a new class of target/effector molecules that utilize both regions of the switch I domain and an insert region distinct to Rho proteins for binding to Cdc42.

The GTP-binding protein Cdc42 was first discovered in Saccharomyces cerevisiae, where it has been shown to have an essential role in bud site assembly, presumably through effects on the actin cytoskeleton (1, 2). In mammalian cells, it has been suggested that activation of Cdc42 represents the first step in a GTPase cascade that regulates cytoskeletal changes. Specifically, Cdc42 first stimulates the formation of actin-containing filopodia, and then activation of Rac stimulates the formation of lamellipodia. Finally, activation of RhoA promotes the generation of actin stress fibers (3-6). Recently, it has also been shown that both Cdc42 and Rac stimulate DNA synthesis as well as initiate a protein kinase cascade that begins with activation of the p21-activated serine/threonine kinases (PAKs) and culminates in the stimulation of the nuclear mitogen-activated protein kinases, the c-j un kinase (JNK1), and p38 (7-10, 37).

During the past several years, a good deal of information has been obtained regarding the identity of proteins that regulate the GTP-binding/GTPase cycle of Cdc42. The Dbl oncoprotein was first reported to stimulate the guanine nucleotide exchange activity of Cdc42 and is now known to be a prototype for a family of growth-regulatory proteins (11, 12). A second regulator, called the GDP dissociation inhibitor, opposes the activity of Dbl by inhibiting guanine nucleotide exchange activity (13) but also is capable of stimulating the release of Cdc42 from membranes (13) and inhibiting GTP hydrolysis (14). A third class of regulators of Cdc42 is the family of GTPase-activating proteins or GAPs (15-18).

At present, it is not known why there are so many potential regulators for the GTP-binding/GTPase cycle of Cdc42 or whether all of these regulators are important in vivo. However, there is increasing evidence that Cdc42 stimulates multiple signaling pathways that impact on the nucleus, the cytoskeleton, and, potentially, intracellular trafficking (19, 20). Thus, Cdc42 may require a number of regulatory factors to ensure proper GTP binding/GTPase cycling at different locations as well as a number of different target molecules to mediate its diverse biological activities. Probably, the best known class of targets are the members of the PAK family (21-23), whose serine/threonine kinase activities are strongly stimulated by GTP-bound Cdc42 and are involved in signaling pathways that culminate in the activation of the nuclear mitogen-activated protein kinases c-j un kinase and p38. However, thus far, there is no indication that the PAK’s are essential for other aspects of Cdc42 signaling, which has led us, as well as other groups, to search for additional target/effecter molecules for Cdc42.

In order to identify new candidate Cdc42 targets, we have used glutathione S-transferase (GST)-Cdc42 fusion proteins, immobilized on glutathione-agarose beads, as affinity resins. In the present work, we describe two proteins from rabbit liver cytosol that bind to Cdc42. One of these, molecular mass ~180 kDa, binds preferentially to the GTP-S-bound form of Cdc42 and has all the hallmarks of a target molecule. The second, molecular mass ~175 kDa, associated with both nucleotide-bound and nucleotide-free forms of Cdc42. Both of these proteins were purified and sequenced. The ~180-kDa protein was
shown to share a high degree of sequence similarity with a recently identified protein that contains a putative calponin domain and an IQ (calmodulin-binding) domain as well as a RasGAP domain and has been designated IQGAP1 (24, 25). The ~175-kDa protein showed high sequence similarity to another RasGAP-related protein, IQGAP2, which appears to be very closely related to IQGAP1 and shares all of the same features.

**MATERIALS AND METHODS**

Preparation of Rabbit Liver Cytosol—Livers were removed from freshly exsanguinated Flemish Giant rabbits, which were used for the production of antibodies at the Cornell Veterinary School, and placed in ice-cold 250 mM sucrose buffered with 100 mM Tris, pH 7.2. The liver was cut into small pieces in a 1:5 (w/v) mixture of the sucrose/Tris solution with the addition of 200 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml aprotinin on ice and then homogenized with a C-Thomas Teflon/glass homogenizer. The homogenate was centrifuged for 10 min at 10,000 x g, and the pellet was discarded. The supernatant was centrifuged at 105,000 x g for 90 min, and the supernatant from this centrifugation was used as “cytosol” in these experiments.

Preparation of Cdc42 Mutant Constructs, GAPC234, and p21-binding Domain (PBD)—The Cdc42-Ras chimera (designated “flapless”), which was prepared by the overlapping polymerase chain reaction method using pET15b vector primers (sense, 5′-CTA TAG GGA ATT TGT GAG GGG-3′; antisense, 5′-GCC ACC TGA CGT CTA AGA AAC-3′) and internal primers (sense, 5′-GCT GCT ACC GTG GAG GCC ACT GCC CCT ACC-3′; antisense, 5′-GGA GAA CCG GTT ACC ATG TGT ACC CCC AAC AAC-3′). The construction of the PBD from pMAP-3 was performed as described (10). The construction of Cdc42 effector mutants T35A, D38E, and Y32K was performed essentially as described for mutants in the Ras effector loop and as previously reported (26). The 234-amino acid catalytic domain of the Cdc42-GAP was provided by Drs. E. Barford and Avi Ashkenazi (Genentech Inc., Emeryville, CA) in pGEX-3X (15), and the catalytic domain of the Cdc42-GAP was provided by Drs. E. Barfod and D. Barford (24, 25). The construction of Cdc42 effectormutants T35A, D38E, AAGC-3′, and Y32K was performed essentially as described for mutants in the Ras effector loop and as previously reported (26).

Preparation of Fusion Proteins—Recombinant proteins containing GST fused to Cdc42, Cdc42(Q61L), Cdc42(Y32K), Cdc42(T35A), Cdc42(D38E), GAPC234, RhoA, Rac, and Ras were expressed in E. coli. GST-Cdc42, GST-Cdc42(Q61L), GST-Cdc42(Y32K), GST-Cdc42(T35A), and GST-Cdc42(D38E) were expressed in pET15b vector primers (sense, 5′-CTA TAG GGA ATT TGT GAG GGG-3′; antisense, 5′-GCC ACC TGA CGT CTA AGA AAC-3′) and internal primers (sense, 5′-GCT GCT ACC GTG GAG GCC ACT GCC CCT ACC-3′; antisense, 5′-GGA GAA CCG GTT ACC ATG TGT ACC CCC AAC AAC-3′). The construction of the PBD from pMAP-3 was performed as described (10).

Affinity Precipitation Experiments—GST-Cdc42 or other fusion proteins were loaded with nucleotide as follows. 15 µl of glutathione-agarose beads containing GST protein which was incubated with either 30 µM GTP, GDP, or GTP and 5 mM MgCl₂ at 30 min at room temperature. Nucleotide-depleted samples were treated with 10 mM EDTA and incubated as above. The glutathione-agarose beads were then pelleted with 400 µl of 2.5 mg/ml cytosol in 0.5% Triton X-100, 5 mM MgCl₂, 100 mM NaCl, and 20 mM Tris-HCl, pH 7.2. In some cases, cytosol was preincubated with glutathione-agarose beads containing GST alone (20 µg/5 mg of cytosolic protein) to reduce nonspecific binding. EDTA was added to nucleotide-depleted samples to give a final concentration of 5 mM. Samples were incubated at 4°C for 1.5 h with rocking, and then beads were pelleted by brief microcentrifugation and washed three times with 100 µl of 0.1% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl, pH 7.2, and then either 5 mM MgCl₂ or 5 mM EDTA. Beads were boiled in 30 µl of sample buffer and run on 8% SDS-PAGE gels. Proteins were visualized by silver staining.

Identification of Cdc42-binding Proteins Using GST-Cdc42 as an Affinity Reagent—In order to identify proteins in rabbit liver fractions that represent new candidate target/effector molecules for Cdc42, we used a GST-Cdc42 fusion protein, immobilized on glutathione-agarose beads, as an affinity reagent.

**RESULTS**

Identification of Cdc42-binding Proteins Using GST-Cdc42 as an Affinity Reagent—In order to identify proteins in rabbit liver fractions that represent new candidate target/effector molecules for Cdc42, we used a GST-Cdc42 fusion protein, immobilized on glutathione-agarose beads, as an affinity reagent. The GST-Cdc42 was loaded with either GTP, GDP or depleted of guanine nucleotide by the addition of EDTA and then incubated with rabbit liver cytosol for 15 h at 4°C, followed by precipitation with glutathione-agarose and SDS-PAGE. Fig. 1A shows that following silver staining, two specific bands were observed that were absent when control experiments were performed using GST alone. One of these, which had an apparent molecular mass of 180 kDa (designated from here on as p180), associated preferentially with the GTPγS-bound state of Cdc42 and did not bind to the nucleotide-depleted form of Cdc42. Occasionally, we observed weak binding of p180 to Cdc42 in the GDP-bound state (data not shown). A second band with an apparent size of 175 kDa (designated p175) appeared to associate with each of the different nucleotide forms of Cdc42.

Fig. 1B shows the results of a similar experiment, where we compared the abilities of GST-Cdc42 preloaded with GTPγS or GTP to bind p180. Previously, we had shown that Cdc42 has a...
relatively fast intrinsic GTP hydrolytic rate (16), which has often made it difficult to use GST-Cdc42-GTP as an affinity reagent for identifying target molecules (26). However, we found that even when GST-Cdc42-GTP was incubated with lysates containing p180 for up to 2 h, it was still possible to precipitate a Cdc42-GTP-p180 complex. These results suggest that p180 maintains Cdc42 in an activated, GTP-bound state and that, as in the cases for the GDP dissociation inhibitor that p180 maintains Cdc42 in an activated, GTP-bound state and that, as in the cases for the GDP dissociation inhibitor molecule (14) and the target PAK (21), p180 acts as a GTPase inhibitor.

We next examined the specificity of p180 and p175 for binding to Cdc42 versus related GTP-binding proteins. Fig. 2 shows that both p180 and p175 can bind to Rac1, which is 70% identical to Cdc42 (27), with p180 and p175 both binding preferentially to the GTP-S-bound state of the GTP-binding protein. However, when directly comparing Cdc42 and Rac1, we find that both p180 and p175 bind with apparent higher affinity to Cdc42, such that at lower levels of the GTP-binding proteins it is possible to detect an association with Cdc42 under conditions where no binding to Rac is observed (data not shown). Neither p180 nor p175 shows any detectable association with RhoA or Ha-Ras, even when using relatively high levels of these GTP-binding proteins.

Competition Studies—Since the binding of p180 was strongest for the GTP-bound form of Cdc42, we next examined whether other proteins that bind preferentially to the activated state of Cdc42 could compete with p180 in this assay. We first examined a truncated form of the Cdc42-GAP (15, 16) that contains 234 amino acids (GAP234) and has been shown to be fully sufficient for binding Cdc42 and for catalyzing the GTPase reaction.2 As shown in Fig. 3A, when the GAP234 was added to rabbit liver cytosol in concentrations as high as 100 μM (which is well above the apparent Kₐ for GAP234 interactions with GTP-S-bound Cdc42, i.e. ~0.5 μM)² and then incubated with the GST-Cdc42-GTPₘ₅, there was no significant decrease in the amount of p180 that associated with Cdc42.

In order to be certain that GAP234 was able to bind the GST-Cdc42 fusion protein and catalyze GTP hydrolysis, we performed control experiments using GTP-bound GST-Cdc42. We reasoned that if the GAP were able to functionally couple to GST-Cdc42, it would catalyze GTP hydrolysis and convert the GTP-bound Cdc42 to the GDP-bound species and thus significantly decrease its ability to bind p180. We, in fact, observed that preincubation of GST-Cdc42-GTP with GAP234 completely eliminated the ability of Cdc42 to subsequently bind p180 (data not shown). Thus, these results indicate that the limit GAP-domain is able to bind and functionally couple to GTP-bound GST-Cdc42; however, the binding site for the GAP must not overlap the binding site for p180.

We next examined whether a known target for Cdc42, mPAK-3 (22), competed with p180 for binding to GTP-S-bound Cdc42. It was originally suggested that a region of ~60 amino acids within the amino-terminal domain of the PAKs was responsible for binding to Cdc42 and/or Rac (21), and we recently found that this limit domain, designated PBD, contains all the determinants necessary for full binding by mPAK-3 to Cdc42.³ As shown in Fig. 3B, we found that, unlike the case for the Cdc42-GAP, the PBD effectively competed with p180 for GTP-S-bound Cdc42 and to a lesser extent for p175. At concentrations as low as 5 μM, p180 binding was completely eliminated, whereas the binding of p175 was weakened in the presence of 10 μM PBD. These data then suggest that the mPAK-3 target molecule shares some portion of the p180 and p175 binding sites on Cdc42.

Characterization of the Abilities of Different Cdc42 Mutants to Bind to p180 and p175—Given the results from the competition experiments described above and the fact that p180 has a higher affinity for activated forms of Cdc42, we examined the abilities of both p180 and p175 to bind to Cdc42 molecules that contained mutations within the predicted switch I target/effector-binding domain (28, 29). Three such mutants that we examined were Cdc42(D38E), Cdc42(Y32K), and Cdc42(T35A). In each case, the analogous mutation in Ras eliminates its ability to bind both to the RasGAP and to Ras target molecules. However, as shown in Fig. 4A, the Cdc42(D38E) mutant had no effect on the binding of either p180 or p175. The Cdc42(Y32K) mutation was capable of little or no binding to p180, but the GTP-S-bound form of Cdc42(Y32K) still showed some ability to bind p175. Binding of p175 to this Cdc42 mutant was typically less effective than for wild type Cdc42, and the GDP-bound form of Cdc42(Y32K) showed no ability to bind p175.

The Cdc42(T35A) mutation was completely ineffective in binding either p180 or p175. The substitution of an alanine for a threonine at position 35 is predicted to destabilize the binding of Mg²⁺ to Cdc42 and thus weaken high affinity binding of GTP. Whereas it would be expected that this mutation would disrupt the binding of p180, it was surprising to find that it also inhibited the binding of p175, since this protein appeared to bind to Cdc42 in a nucleotide-independent manner. Thus, it is

FIG. 2. Association of p180 and p175 with Rac1, RhoA, or Ha-Ras proteins. GST fusion proteins of Rac1, RhoA, and Ha-Ras were immobilized on glutathione-agarose beads, loaded with nucleotide, and used in precipitation experiments as described in the legend to Fig. 1A. Association of p180 and p175 (*) was determined by silver stain analysis of SDS-PAGE gels. GST alone on beads was used as a control.

FIG. 3. Competition with p180 and p175 by Cdc42-GAP and mPAK-3. A, a GTPase-defective Cdc42 mutant (GST-Cdc42(L61)) or GST alone as a control was used to precipitate the p180 and p175 proteins (*) from rabbit liver cytosol in the presence of increasing amounts of the limit functional domain of the Cdc42-GAP protein (GAP234). Effects on p180 and p175 binding were visualized in the presence of 0, 1, 10, and 100 μM GAP234 by silver stain analysis of SDS-PAGE gels. B, experiments were performed exactly as in A using the Cdc42-binding domain from the PAK kinase, mPAK-3 (PBD), as a competitor at concentrations of 0, 1, 5, and 10 μM.

2 D. Leonard and R. Cerione, unpublished data.

3 D. A. Leonard, R. Satoskar, W. J. Wu, S. Bagrodia, R. A. Cerione, and D. Manor, manuscript in preparation.
When they were loaded with GDP rather than GTP, nor flapless Cdc42 could compete with p180 or p175 binding the GTP-bound forms of wild type Cdc42 and the Cdc42/Ras chimera, which appear to bind with identical affinities to wildtype Cdc42 in competing against GST-Cdc42 for both p180 and p175 proteins.

These results suggested that Ras and Cdc42 have some subtle, although potentially important, differences in their abilities to couple to GAP and target molecules. This became even more apparent when we examined the interactions of p180 and p175 with a Cdc42/Ras chimera (flapless) in which residues 120–126 from Ras were substituted for residues 120–139 from Cdc42 (the Rho family-specific inserted region or flap), were used to compete with immobilized GST-Cdc42 for p180/p175 binding. Experiments with 0, 1, or 5 μM wild type Cdc42 used as the competitor, preloaded with GTP-γ-S (lanes 1, 2, and 3, respectively) or 1 and 5 μM flapless-Cdc42 preloaded with GTP-γ-S (lanes 4 and 5, respectively) were shown. Control experiments in which wild type and flapless Cdc42 were preloaded with GDP are shown in lanes 6 and 7.

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Very recently, a second putative target for Cdc42, the protein involved in Wiskott-Aldrich syndrome (designated WASP), has been identified (30, 31). The WASP protein shares a region of similarity with the PBD of the PAK molecules, and it appears to be this domain that is responsible for specifically binding GTP-bound Cdc42. Based on microinjection experiments, it was proposed that WASP might represent a link between activated Cdc42 and the actin cytoskeleton (32). However, Cdc42 appears to be ubiquitously distributed and has been shown to induce marked cytoskeletal alterations in a variety of cell types, whereas WASP is a hematopoietic-specific protein. Thus, it seems likely that other target molecules for Cdc42 that perform this function in cells that are not of hematopoietic origin must exist.

In the present study, we have identified and biochemically characterized two proteins from rabbit liver cytosol, molecular mass ~180 and 175 kDa, that represent a potentially new class of Cdc42 targets. Microsequence analysis indicates that the 180-kDa protein is most likely the rabbit liver IQGAP1 protein, whereas the 175-kDa protein appears to be IQGAP2. Early interest in the IQGAP proteins stemmed from the fact that they contained a domain that shared significant sequence homology with the active site of RasGAP. However, recently, the IQGAP1 protein was identified in COS cell lysates as a potential target for Cdc42 but not Ras (25). Similarly, we have found no indication for Ras binding to the rabbit liver p180/IQGAP1 nor to p175/IQGAP2. The fact that the GTP-bound state of Cdc42 appears to bind to p180/IQGAP1 preferentially and the finding that the binding of p180/IQGAP1 preserves the activated state of Cdc42 suggest that p180/IQGAP1 is a target/effector molecule for Cdc42. Further support for this suggestion comes from the finding that the Cdc42-target mPAK-3 competes with p180/IQGAP1 for Cdc42 and from the fact that switch I effector domain mutations in Cdc42 affect the binding of p180/IQGAP1. Moreover, we have recently been able to monitor a direct interaction between Cdc42Hs and the carboxyterminal half of the IQGAP1 molecule (residues 918-1657) that was expressed in insect cells and purified as a Glu-Glu-tagged fusion protein. This amino-terminal truncated IQGAP1 molecule, which still contains the RasGAP homology domain, binds specifically to Cdc42Hs that contains the GTP analog, Mant-GMP-PNP, but not to Cdc42Hs containing Mant-GDP. The binding of the recombinant IQGAP1 molecule to Mant-GMP-PNP·Cdc42Hs results in an ~3-fold enhancement of the N-methylanthraniloyl fluorescence with an apparent K_{d} of ~50 nM.

At present, it is not entirely clear whether IQGAP2 also represents a true candidate target/effector for Cdc42, mainly because the binding of the cytosolic p175/IQGAP2 molecule did not show a marked dependence on guanine nucleotide. However, it does appear, that under some conditions, p175/IQGAP2 prefers the GTP-bound Cdc42 over the GDP-bound form. For example, exogenously added Cdc42-GDP is unable to compete with immobilized Cdc42-GTP·S for p175 binding. In addition, most other aspects of the interaction between p175/IQGAP2 and Cdc42 are very similar to those for p180/IQGAP1, and the overall sequence similarity between IQGAP1 and IQGAP2 leads us to suspect that these two proteins are likely to mediate similar activities.

Clearly, an important question concerns the physiological function of the IQGAP molecules. Given the sequence homology between the IQGAPs and two cytoskeletal proteins, myosin and calponin, one possibility is that the IQGAP molecules serve as some type of interface between activated Cdc42 and cytoskeletal proteins. However, analyses of the abilities of Cdc42 mutants to bind to p180/IQGAP1 and p175/IQGAP2 when compared with the effects of mutated Cdc42 molecules on filopodia formation would seem to suggest that the IQGAPs are not critical targets for this cytoskeletal change. Specifically, while Cdc42 proteins mutated at position 38 (normally an aspartic acid) are still able to bind to p180/IQGAP1 and p175/IQGAP2, Best et al. (33) have reported that a Cdc42(D38A) mutant does not stimulate filopodia formation when it is microinjected into cells. Another possibility that we are currently pursuing is that an IQGAP molecule may be involved in some aspect of Cdc42 function in the Golgi apparatus. Both indirect immunofluorescence experiments and cell fractionation studies indicate that a predominant localization of Cdc42 is in the Golgi membrane (20) and recent work by Singer et al. (19) suggests that Cdc42 is capable of acting synergistically with Arf to stimulate a Golgi membrane-associated phospholipase D enzyme. We have recently identified an ~175-kDa protein in Golgi membranes that binds selectively to GTP·S-bound Cdc42 and shares many characteristics with the cytosolic IQGAP molecules described in this study. If it turns out that this Golgi membrane protein is in fact a member of the IQGAP family, it would suggest a number of interesting possibilities regarding a possible role for IQGAP in the recruitment of activated Cdc42 molecules to Golgi membranes and/or an involvement of IQGAP in coupling transport events that occur in or originate from the Golgi apparatus with cytoskeletal activities.

Finally, an interesting and somewhat unexpected outcome of the work presented here concerns the possible involvement of an insert region on Cdc42, which is unique to the Rho subfamily proteins, in the binding of targets. Based on the results from competition experiments, we conclude that the Cdc42Hs·GAP and p180/IQGAP1 bind to distinct sites on activated Cdc42 molecules, whereas mPAK-3 binds to activated Cdc42 at a site(s) that overlaps the binding domain for IQGAP proteins. This is somewhat reminiscent of the situation for Ras, where it has been elegantly demonstrated that different targets appear to bind to distinct portions of the switch I domain, based on the fact that certain “effector loop” mutations of Ras inhibit the binding of one but not all targets (34). We suggest that the insert region on Cdc42 potentially represents an additional target-binding domain, because its removal appears to markedly weaken the binding of the IQGAP molecules. The fact that the removal of this insert region is without any apparent effect on the binding of mPAK-3 further suggests that this region may be important for binding some but not all Cdc42 targets. The IQGAP molecules may require both the insert region and some residues within the switch I domain for proper binding, thereby accounting for the competition between p180/IQGAP1 (or p175/IQGAP2) and mPAK-3 for binding to activated Cdc42. Apparently, the Cdc42·GAP molecule binds to residues within the switch I domain that are not important for binding the IQGAP molecules but are involved in binding mPAK-3, thus accounting for the observed competition between GAP and mPAK-3 for Cdc42. The potential involvement of the insert region or “flap” in binding certain targets of Cdc42 now opens the way to some interesting experiments regarding the examination of the effects of Cdc42 molecules that lack this region on various biological activities including cell growth and filopodia formation.

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