Identification of an Actin Cytoskeletal Complex That Includes IQGAP and the Cdc42 GTPase*

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The Rho subfamily of low molecular weight GTPases have been implicated in a variety of cellular functions that include reorganization of the actin cytoskeleton and stress-induced activation of the c-Jun kinase. The downstream targets that mediate the effects of Cdc42 on the actin cytoskeleton have yet to be fully identified. We have used the transient transfection of COS-7 cells with epitope-tagged Cdc42 to identify candidate signaling partners for this GTPase and identified the IQGAP protein as a major in vivo target for activated Cdc42. Epidermal growth factor stimulation of serum-starved COS-7 cells promoted the formation of a Cdc42-IQGAP complex, indicating that growth factors can increase the pool of activated Cdc42. Activated HA-Cdc42 co-localized with IQGAP or F-actin in vivo, whereas cells transfected with dominant-negative forms of Cdc42 (Cdc42(T17N)) showed predominantly dispersed distributions for both HA-Cdc42 and endogenous IQGAP. In detergent lysates from COS-7 cells transiently transfected with different forms of Cdc42, or from stably transfected CHO cells, the induction of actin polymerization by phallloidin resulted in the incorporation of both IQGAP and Cdc42 into actin-containing complexes. Taken together, these findings are consistent with a model whereby IQGAP serves as a target for GTP-bound Cdc42 providing a direct link between the activated GTPase and the actin cytoskeleton.

Accumulating evidence indicates that members of the Rho subfamily of low molecular weight GTPases play pivotal roles as molecular switches by controlling the dynamics of actin assembly (1–10). Microinjection of activated forms of Rho, Rac, and Cdc42 give rise to changes in the underlying actin structures of cells, as manifested by an increase in the number and extent of actin stress fibers (Rho (11)), lamellipodia (Rac (12)), and filopodia (Cdc42 (2, 13)). Despite numerous studies using microinjection and genetic approaches to demonstrate the importance of Rho, Rac, and Cdc42 in these processes, little is known about the protein-protein interactions that give rise to these morphological changes.

To identify specific targets for Cdc42, which may play critical roles in mediating the effects of this GTPase on the actin cytoskeleton and cell morphology, we have used epitope-tagged mutant forms of Cdc42 to facilitate co-immunoprecipitation and co-localization studies in transfected cell populations. In this study, co-immunoprecipitation experiments demonstrate that a major target of the activated form of Cdc42 in mammalian cells is IQGAP1, a previously described protein possessing a Ras-GTPase activating domain (3). We show that Cdc42 and IQGAP associate in response to the addition of EGF, demonstrating the activation of cellular Cdc42 via growth factor-stimulated pathways. Cdc42 is seen to co-localize with endogenous IQGAP and actin in transfected COS-7 cells, giving rise to numerous pleated structures, and to co-immunoprecipitate with these proteins in an F-actin- and GTP-dependent manner. Our results suggest that the assembly of a stable complex containing Cdc42, IQGAP, and F-actin is regulated by the GTPase activity of Cdc42.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The affinity-purified antibody recognizing IQGAP1 was raised in rabbits injected with the amino-terminal half of Sf9-expressed IQGAP1 as described previously (4). The monoclonal antibody 12CA5 specifically recognizing the hemagglutinin (HA) peptide YYDYVPDYA was purchased as mouse ascites from Berkeley Antibody Corp. Anti-actin (rabbit) antibody was purchased from Sigma. Other chemicals were purchased as standard reagent grade and were obtained from Sigma unless otherwise noted.

Cell Culture and Transfection—COS-7 cells (ATCC) were grown in DMEM supplemented with 10% fetal calf serum and plated overnight prior to transfection. Plasmids encoding mutant forms of Cdc42 were constructed by ligating the BamHI-EcoRI Cdc42 coding sequence from pGEX-KG into the mammalian transfection plasmid pKH2 (a generous gift of I. Macara, University of Virginia) containing the oligonucleotide coding region for three tandem amino-terminal hemagglutinin epitope sequences as described previously (5). COS-7 cells were transiently transfected with purified pHKH2/Cdc42 plasmid preparations using the LipofectAMINE™ protocol as per manufacturer's instructions (Life Technologies, Inc.). Approximately 24 h post-transfection, cells were trypsinized and replated onto glass coverslips (22 × 22 mm; Corning) and assayed between 48 and 72 h post-transfection. CHO cells were stably transfected with the wild-type pHKA3 construct of Cdc42 at a 10:1 ratio with a plasmid encoding G418 resistance.

[35S]Methionine metabolic labeling was carried out using 20 mCi of Tran35S-label™ on 100-mm tissue culture plates of transfected COS-7 cells (24 h post-transfection) for 15 h in 4 ml of methionine/cysteine-free DMEM supplemented with 10% fetal calf serum. After 15 h of labeling, cells were rinsed in PBS (10 mM KH2PO4, pH 7.4, 120 mM NaCl), harvested in lysis buffer (20 mM HEPES, pH 7.4, 120 mM NaCl, 30 mM KCl, 5 mM MgCl2, 1% Nonidet P-40, 10 mg/ml each of leupeptin and aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride), and treated as described below for non-radioactive immunoprecipitations.

Immunoprecipitation and Western Blotting—Transfected COS-7 cells were lysed 48–72 h post-transfection (1 × 106 cells/35-mm well) in ice-cold detergent lysis buffer for 10 min at 4 °C. Lysates were transferred to pre-chilled tubes and centrifuged at 15,000 × g for 10 min at 4 °C to remove detergent-insoluble material. Supernatants were rocked for 2 h (4 °C) after addition of mouse ascites containing anti-HA antibody. Immune complexes were isolated by adding 20 ml of suspended

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1 The abbreviations used are: EGF, epidermal growth factor; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GTP, γ-32P, guanosine 5′-O-(3-thiotriphosphate).
protein A-Sepharose beads (100 mg of swollen beads/ml) to each tube and rocking for 2 h. Samples were then centrifuged at 15,000 \( \times g \) for 2 min. Beads were washed three times in lysis buffer for 10 min each before resuspension of the beads in 50 ml of SDS sample buffer. Samples were resolved by SDS-PAGE on 12.5% acrylamide gels and transferred overnight to Immobilon-P membranes (Millipore). After blocking with 2% dry milk in TBS-Tween (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% Tween) for 1 h, membranes were probed with primary antibodies as indicated. After 3 washes of 30 min each in TBS-Tween, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000 in TBS-Tween buffer), washed 3 times with 20 ml of TBS-Tween, and visualized with ECL (Amersham).

**Immunocytochemistry**—Transfected COS-7 cells were replated 24 h post-transfection onto glass coverslips and allowed to adhere (for 24–48 h) before fixation for 10 min with 3.7% formaldehyde in PBS. Following 3 washes with PBS (10 min each), cells were permeabilized for 5 min in PBS-containing 0.1% Triton X-100 and washed three times in PBS. For indirect immunofluorescence of epitope-tagged Cdc42, a monoclonal anti-HA antibody (12CA5) was used 1:100. For localization of endogenous IQGAP, a polyclonal antibody raised against the amino terminus of recombinant IQGAP1 was used at a dilution of 1:50. Diluted primary antibodies were incubated with the fixed and permeabilized cells for 2 h at 22 °C. After washing the primary antibodies for 30 min with PBS, secondary antibodies (Molecular Probes) conjugated to rhodamine (anti-mouse, 1:200 dilution) or Bodipy® (anti-rabbit; 1:100 dilution) fluorophores were incubated for 1 h at room temperature prior to the mounting of coverslips with non-fade medium (Kirkegaard and Perry, Gaithersburg, MD). F-actin was visualized by incubating fixed and permeabilized cells with rhodamine phalloidin (1:20 dilution of a 1 mg/ml stock in ethanol; Molecular Probes) in the secondary incubation step.

**In Vitro Actin Complex Formation**—Cells expressing HA-tagged Cdc42 were resuspended and lysed at 1 \( \times 10^6 \) cell eq/ml with 10 strokes of a 50-ml glass homogenizer in actin polymerization buffer (150 mM KCl, 20 mM HEPES, pH 7.4, 2 mM MgCl\(_2\), 2 mM K\(_2\)HPO\(_4\), 1% Nonidet P-40, 10 \( \mu \)g/ml each aprotinin and leupeptin). Following a 10-min incubation on ice, the lysate was spun for 90 min at 170,000 \( \times g \) at 4 °C in a Beckman type Ti-50 fixed angle rotor. The supernatant was aliquoted into chilled Microfuge tubes, 1 ml or 10\(^6\) cell eq/tube, and each was made 25 \( \mu \)M in ATP by additions from a 5m M stock solution. The supernatant was spun for 90 min at 170,000 \( \times g \) at 4 °C. Anti-HA ascites (3 \( \mu l \) each sample) was added, and the samples were rocked an additional 2 h at 4 °C prior to isolation of the immune complexes with protein A-Sepharose beads. These pellets were washed three times in actin lysis buffer at 4 °C, and the final pellet was resuspended in SDS-PAGE sample buffer. Samples were boiled for 5 min and resolved by gel electrophoresis as described above.

**RESULTS AND DISCUSSION**

**Co-immunoprecipitation of IQGAP1 with Activated Cdc42**—Co-immunoprecipitation of proteins specifically bound to the amino-terminally HA-tagged forms of Cdc42 was carried out in transfected COS-7 cells expressing wild-type (WT), dominant-negative (T17N) or constitutively active (Q61L) Cdc42, or vector alone. Biosynthetic labeling of cellular proteins in these transfected cells with \[^{35}S\]methionine, followed by immunoprecipitation, revealed an intense band at 195 kDa in autorigraphed gels that co-precipitated with the constitutively active form of HA-Cdc42 (Fig. 1A). Low levels of p195 co-immunoprecipitated with the wild-type form of HA-Cdc42 whereas no association between p195 and HA-Cdc42-T17N was detected even when the dominant-negative form of HA-Cdc42 was expressed at higher levels. The interaction between p195 and HA-Cdc42 was specific for HA-Cdc42, as HA-tagged Rho and Rac showed no detectable co-immunoprecipitation with p195 (data not shown).

The \[^{35}S\]methionine-labeled p195 protein was identified by Western blotting with an antibody specific for IQGAP1. Antibodies raised against the amino- and carboxyl-terminal halves of recombinant IQGAP1 both recognized immunoprecipitated p195 (data not shown). Previous work by us (4, 6) had shown that glutathione S-transferase-Cdc42-GTPyS complexes bind IQGAP in a variety of cell extracts, further suggesting that this protein is a target for activated forms of Cdc42. A second co-immunoprecipitated protein of \( \sim 17 \) kDa has been previously identified as Ca\(^{2+}\)-calmodulin (4). The putative calmodulin binding domain in the IQGAP sequence (i.e. the IQ repeat motif) most likely accounts for the presence of calmodulin in immunoprecipitates of the epitope-tagged Cdc42. The association of calmodulin with immunoprecipitated IQGAP is not stoichiometric; however, it has recently been reported that calmodulin can influence IQGAP interactions with Cdc42 (19) and F-actin (20).

IQGAP and F-actin have previously been shown to co-localize in the peripheral cell cortex (4), and this co-localization has been suggested to be responsive to receptor tyrosine kinase pathways (7). The result of EGF stimulation of serum-starved, transfected COS-7 cells is shown in Fig. 1B. Co-immunoprecipitation of IQGAP with epitope-tagged Cdc42 increased after a 10-min stimulation of EGF. The time course for this increase mirrored that for EGF receptor phosphorylation (bottom of four
panels; Fig. 1B). These results suggest that the EGF receptor kinase activity initiates a signaling pathway leading to the activation of the Cdc42 protein.

**Co-localization of Activated Cdc42 with IQGAP and F-actin**—The specificity and the relatively high affinity of GTP-bound Cdc42 for IQGAP suggested that these two proteins might co-localize in vivo. Fig. 2A shows the immunostaining pattern obtained with anti-HA antibody (red) and antibody recognizing the endogenous IQGAP (green). The top two panels show that a diffuse distribution is observed for the dominant-negative form of HA3-Cdc42T17N and that, in the HA3-Cdc42T17N transfected cells, a similarly diffuse distribution is observed for the endogenous IQGAP. In contrast, transfection of COS-7 cells with GTPase-defective HA3-Cdc42Q61L resulted in a significant overlap between HA-Cdc42 and IQGAP in the form of large pleats which included both proteins (bottom panels; Fig. 2A). A significant percentage of cells expressing the HA-tagged wild-type Cdc42 also displayed the pleated structures (data not shown). This result is consistent with the view that Cdc42 acts upstream of IQGAP, perhaps by directing its incorporation into Cdc42-IQGAP complexes.

We then examined whether F-actin was associated with these complexes. In cells staining positively for transfected, HA-tagged Cdc42WT and HA-Cdc42Q61L, we observed pleated structures that were shown to also contain F-actin, as these filaments stained with rhodamine phalloidin (14) (Fig. 2B). In experiments using three different fluorescent dyes well resolved in their respective excitation and emission wavelengths, all three proteins, HA-Cdc42, IQGAP, and actin were seen to co-localize (data not shown), suggesting that they participate in the formation of a specialized, Cdc42-regulated structure.

**Biochemical Evidence for a Complex That Includes Cdc42, IQGAP, and F-actin**—It has recently been reported that IQGAP purified from bovine adrenal binds to F-actin (20). We were interested in obtaining biochemical evidence for a Cdc42-IQGAP-F-actin complex. Lysates were prepared from cells transfected with wild-type HA-Cdc42, dominant-negative HA-Cdc42T17N, or constitutively active HA-Cdc42Q61L. The lysates were cleared of F-actin after centrifugation and then incubated with 25 μM phalloidin (a concentration that exceeds the remaining actin concentration as estimated by a comparison of immunoblotted actin standards (data not shown)). While the HA-Cdc42Q61L species remained capable of co-immunoprecipitating IQGAP (top panel, lane 5, Fig. 3A), this was not observed for the phalloidin-treated sample (lane 6). Although the endogenous Cdc42 in transfected COS-7 lysates is about equimolar with the transfected HA-Cdc42Q61L,
this last result suggests that endogenous wild-type Cdc42 participates more effectively than the transected Cdc42\textsuperscript{Q61L} in the formation of stable IQGAP-actin complexes in the presence of phallolidin, thereby depleting the endogenous IQGAP pool and rendering it unavailable for co-immunoprecipitation with Cdc42\textsuperscript{Q61L}. One possible alternative explanation for these data may include IQGAP-F-actin guanine nucleotide exchange activity, which leads to the preferred incorporation and activation of wild-type Cdc42. We feel that it is unlikely that either F-actin or IQGAP alone is capable of guanine nucleotide exchange activity because we have not found F-actin to directly bind to Cdc42 nor have we detected exchange activity in purified preparations of IQGAP (data not shown). Nevertheless, the possibility that actin-IQGAP complexes in the phallolidin-treated lysates may serve as points of recruitment for other proteins including endogenous nucleotide exchange factors remains an intriguing possibility that warrants closer examination.

Under the conditions of this experiment, the wild-type HA-tagged Cdc42 did not co-immunoprecipitate detectable IQGAP in the control lane (lane 1, Fig. 3A). Only after the addition of the F-actin stabilizing compound, phallolidin, did the centrifuged lysate yield a complex containing HA-tagged wild-type Cdc42 together with IQGAP and actin. These results indicate that Cdc42 is part of a multimeric actin complex. In contrast, HA-Cdc42\textsuperscript{T17N} was not capable of co-immunoprecipitating either IQGAP or actin under conditions where the F-actin pool was increased by the addition of phallolidin (Fig. 3A, lanes 3 and 4), consistent with the findings from immunofluorescence experiments that HA-Cdc42\textsuperscript{T17N} does not co-localize with F-actin or IQGAP.

The requirement for wild-type Cdc42 suggests that the GTP hydrolytic activity and therefore the cycling of the GTPase between active and inactive states is necessary for the formation of a stable ternary complex, Cdc42-IQGAP-F-actin. Additional support for this view was obtained from studies with a stably transfected cell line expressing the wild-type HA-tagged Cdc42 construct. The transected CHO cells provided a homogeneous cell population and led to better reproducibility in the actin polymerization experiments. After clearing these lysates of any F-actin by centrifugation, 1-ml aliquots of supernatant (1 × 10⁶ cell eq) were tested for HA immunoprecipitation of actin complexes in response to a variety of treatments. The Western blot analysis shown in Fig. 3B is consistent with a role for Cdc42-catalyzed GTP hydrolysis in the formation of Cdc42-IQGAP-actin complexes. Only when GTP was present in a lysate containing phallolidin was an appreciable amount of actin co-immunoprecipitated with the anti-HA (12CA5) antibody (lane 4, Fig. 3B). Significantly, the co-immunoprecipitation of actin was blocked by the non-hydrolyzable GTP analog, GTP\textsuperscript{S}, even while the amount of co-immunoprecipitated IQGAP in this sample remained comparable (lane 5, Fig. 3B). Thus, under the conditions used in these immunoprecipitation experiments where the protein contents of the cell are diluted, added GTP can drive complex formation. The situation may not be so clear-cut in the intact cell where the relevant protein concentrations are ~4 orders of magnitude higher. Immunolocalization of the constitutively active mutant of HA-Cdc42 in transfected cells, which binds tightly to IQGAP but is not observed to be a stable element of the actin complex in immunoprecipitation experiments, may not show the strict nucleotide dependence observed in the cell-free system. The reversible interaction of the HA-Cdc42-IQGAP complex with actin most likely occurs through the amino-terminal domain of IQGAP, which harbors a potential binding site for F-actin, similar to those found in α-actinin and filamin (9, 16–18). One attractive hypothesis regarding the role of Cdc42 in the regulation of the actin cytoskeleton is the allosteric regulation of the availability of this site of actin-IQGAP interaction by the binding of activated Cdc42.

Taken together, the in vitro results suggest a scheme (Fig. 4) in which the formation of a ternary complex between GTP-bound Cdc42, IQGAP, and F-actin represents a transient state which precedes the formation of more stable Cdc42-IQGAP-actin aggregates. In this model, formation of F-actin by the addition of phallolidin in the lysates drives the initial formation of the actin-IQGAP-Cdc42 interaction while hydrolysis of GTP increases the stability of the complex such that it persists under the conditions of the immunoprecipitation experiment. This aspect of the model may explain our findings that HA-Cdc42\textsuperscript{Q61L} and actin co-localize in transfected cells (Fig. 2B) but are unable to form a stable ternary complex as detected by immunoprecipitation (Fig. 3A, lane 6). Based on our data demonstrating that only the wild-type form of Cdc42 is competent at driving the formation of these complexes in vitro, we propose that GTP hydrolysis by Cdc42 results in stably cross-linked actin filaments that are observed in our experiments as immunoprecipitable Cdc42-IQGAP-actin complexes. In this view, actin remodeling, as it pertains to Cdc42 activation, occurs as a consequence of GTP hydrolysis by Cdc42, allowing the wild-type Cdc42 protein to regulate the assembly of F-actin into
stable higher order structures. Cdc42T17N, by competitively inhibiting the activation of endogenous Cdc42, would block both the formation of the transient Cdc42-IQGAP-actin ternary complex and the subsequent formation of a stable ternary complex. Conversely, although Cdc42Q61L binds tightly to IQGAP and co-localizes with IQGAP-actin structures, our model proposes that it is not tightly incorporated into the immuno-precipitable F-actin structures due to the requirement for Cdc42-catalyzed GTP hydrolysis.

The apparent need for a GTPase-competent Cdc42 molecule for the assembly and regulation of higher order actin complexes is provocative given that we have found that expression of GTPase-defective forms of Cdc42 in NIH 3T3 cells, while being capable of binding target molecules and stimulating target kinase activities, is nonetheless deleterious to normal cell growth and cytokinesis. Thus, the ability of Cdc42 to initiate a sequence of events that results in actin reorganization may underlie processes that are fundamentally important to cell viability. Current studies are focusing on identifying other cellular participants in these Cdc42/IQGAP-mediated actin re-arrangements and in establishing how these remodeling events may affect other known Cdc42 cellular activities.

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