The Activity of the GTPase-activating Protein CdGAP Is Regulated by the Endocytic Protein Intersectin

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The Rho GTPases RhoA, Rac1, and Cdc42 play a major role in regulating the reorganization of the actin cytoskeleton. We recently identified CdGAP, a novel GTPase-activating protein with activity toward Rac1 and Cdc42. CdGAP consists of a N-terminal GAP domain, a central domain, and a C-terminal proline-rich domain. Here we show that through a subset of its Src homology 3 domains, the endocytic protein intersectin interacts with CdGAP. In platelet-derived growth factor-stimulated Swiss 3T3 cells, intersectin co-localizes with CdGAP and inhibits its GAP activity toward Rac1. Intersectin-Src homology 3 also inhibits CdGAP activity in GAP assays in vitro. Although the C-terminal proline-rich domain of CdGAP is required for the regulation of its GAP activity by intersectin both in vivo and in vitro, it is not necessary for CdGAP-intersectin interaction. Our data suggest that the central domain of CdGAP is required for CdGAP-intersectin interaction. Thus, we propose a model in which intersectin binding results in a change of CdGAP conformation involving the proline-rich domain that leads to the inhibition of its GAP activity. These observations provide the first demonstration of a direct regulation of RhoGAP activity through a protein-protein interaction and suggest a function for intersectin in Rac1 regulation and actin dynamics.

The Rho family of GTPases, including RhoA, Rac1, and Cdc42 regulates multiple cellular processes including proliferation, motility, adhesion, and intracellular membrane trafficking (1, 2). However, the best characterized function for these proteins is the regulation of the actin cytoskeleton in response to extracellular stimuli (3). For example, treatment of Swiss 3T3 fibroblasts with lysophosphatidic acid or bombesin induces RhoA activation leading to the formation of stress fibers and focal adhesion complexes, whereas growth factors activate Rac1 promoting the formation of an actin meshwork at the cell periphery producing lamellipodia and membrane ruffles (4, 5). In the same cells, bradykinin and the cytokines tumor necrosis factor-α and interleukin-1 activate Cdc42 leading to the formation of filopodia at the cell periphery (6–8).

Rho GTPases act as molecular switches that cycle between inactive, GDP-bound and active, GTP-bound states. This GDP/GTP cycle is regulated by at least three families of proteins including guanine nucleotide dissociation inhibitors, guanine nucleotide exchange factors, and GTPase-activating proteins (GAPs) (9). Guanine nucleotide exchange factors activate Rho GTPases by catalyzing the exchange of GDP for GTP in response to extracellular stimuli, whereas guanine nucleotide dissociation inhibitors appear to stabilize the inactive, GDP-bound form of the protein. GAPs enhance the intrinsic GTPase activity of Rho proteins, thereby leading to their rapid conversion to the inactive GDP-bound form.

We recently identified CdGAP (Cdc42 GTPase-activating protein), a novel serine- and proline-rich RhoGAP protein. CdGAP has in vitro and in vivo activity toward both Rac1 and Cdc42 but not RhoA (10). Most proteins of the RhoGAP family contain additional protein- or lipid-binding motifs that likely serve to regulate their GAP activity and/or subcellular localization (11–13) although the role of these domains is still poorly defined. In addition to its N-terminal GAP domain, CdGAP contains a central domain and five consensus Src homology 3 (SH3)-binding sites located within a large C-terminal proline-rich domain whose functions are still unknown (10).

To better understand the role of CdGAP, we sought to identify binding partners of its C-terminal proline-rich domain. This led to the identification of the endocytic scaffolding protein intersectin as a CdGAP-binding partner. Intersectin is a ubiquitously expressed protein involved in clathrin-mediated endocytosis and cell signaling (14–16). It contains two N-terminal Eps15 homology domains, a central helical region, and five C-terminal SH3 domains (termed SH3A-E) (17, 18). Via its Eps15 homology domains, intersectin interacts with epsin, a protein that binds to clathrin and the clathrin adaptor AP2 (19, 20) inducing localization of intersectin to clathrin-coated pits. Through its SH3 domains, intersectin interacts with mSos1, a guanine-nucleotide exchange factor for Ras and Rac1 and with

** The abbreviations used are: GAP, GTPase-activating protein; SH3, Src homology 3 domain; PDGF, platelet-derived growth factor; CdGAP, Cdc42 GTPase-activating protein; GST, glutathione S-transferase; PBS, phosphate-buffer saline; TRITC, tetramethylrhodamine B isothiocyanate; EV, empty vector.

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Cdc42GAP Regulation by Intersectin

Here, we report that intersectin interacts through a subset of its SH3 domains with Cdc42GAP. Cdc42GAP co-localizes with intersectin in platelet-derived growth factor (PDGF)-stimulated fibroblasts. Moreover, intersectin inhibits Cdc42GAP activity in PDGF-stimulated cells and in GAP assays in vitro. Surprisingly, intersectin is still able to interact with a Cdc42GAP mutant lacking the C-terminal proline-rich domain. We found that the central domain of Cdc42GAP is required to mediate Cdc42GAP-intersectin interaction. However, the ability of intersectin to inhibit Cdc42GAP activity requires the C-terminal proline-rich domain. Our data demonstrate that intersectin directly inhibits Cdc42GAP activity, probably through a change of Cdc42GAP conformation involving the C-terminal proline-rich domain.

**EXPERIMENTAL PROCEDURES**

*DNA Constructs*—DNAs encoding for mouse Cdc42GAP N terminus (residues 3 to 662) and C terminus (residues 600–820) were subcloned into pRK5Myc and pCR2.1 expression vectors, respectively, as previously described (10). Cdc42GAP C terminus encoding cDNA was excised from pCR2.1 using NheI and subcloned into NheI-digested pRK5MycCdGAP-Δ322 resulting in pRK5MycCdGAP-ΔK2. Lysine residue missing at position 2 of the protein was added by polymerase chain reaction (PCR) using 5′-CCGGATCCAGTAAGAAAATCGAAGCC and 5′-GAAATTTGACACCCGAGGTTAACGTTT as forward and reverse primers and pRK5MycCdGAP-ΔK2 vector as template. pRK5MycCdGAP was obtained by ligating pRK5MycCdGAP-ΔK2, digested with BamHI/BglII, and dephosphorylated using calf intestine alkaline phosphatase with BamHI/BglII-digested PCR product. Cdc42GAP-ΔPRD and Cdc42GAP-ΔPRD mutants were generated by PCR using pRK5MycCdGAP as a template, 5′-CGCGCGGCCGCCAACTGCTCCAGGAGTGG and 5′-GAAGATCTGGGCATTGATC as forward and reverse primers, respectively. Both PCR products and pRK5MycCdGAP were digested with ApaI and XhoI together. Cdc42GAP-PRD and Cdc42GAP-ΔPRD mutants were generated using the same template, 5′-CCGGATCCAGTAAGAAAATCGAAGCC and 5′-CGGGATCCAGTAAGAAAATCGAAGCC as forward and reverse primers, respectively. Both PCR products and pRK5MycCdGAP vector were digested with BamHI/XhoI and ligated together. pGEX4T3CdGAP-ΔGAP and pGEX4T3CdGAP-PRD were generated by PCR using pRK5MyccCdGAP as a template, 5′-CCGGATCCAGTAAGAAAATCGAAGCC and 5′-CCGGATCCAGTAAGAAAATCGAAGCC as reverse FLAG primers, respectively. Both PCR products and pRK5MycCdGAP vector were digested with BamHI/XhoI and ligated together.

The SH3 domains of intersectin, Nck, Src, amphiphysin I and amphiphysin II, their SH3 domains with CdGAP. CdGAP co-localizes with intersectin in platelet-derived growth factor (PDGF)-stimulated fibroblasts. Moreover, intersectin inhibits Cdc42GAP activity in PDGF-stimulated cells and in GAP assays in vitro. Surprisingly, intersectin is still able to interact with a Cdc42GAP mutant lacking the C-terminal proline-rich domain. We found that the central domain of Cdc42GAP is required to mediate Cdc42GAP-intersectin interaction. However, the ability of intersectin to inhibit Cdc42GAP activity requires the C-terminal proline-rich domain. Our data demonstrate that intersectin directly inhibits Cdc42GAP activity, probably through a change of Cdc42GAP conformation involving the C-terminal proline-rich domain.

*Cells and Antibodies*—COS-7 and Swiss 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics, and maintained at an atmosphere of 10% CO₂. Anti-Cdc42GAP antibodies were obtained by immunization of rabbits with Cdc42GAP proline-rich domain (amino acids 515 to 820) fused to the GST. Anti-Cdc42GAP antibodies were used at 1/500 dilution in immunofluorescence. Mouse monoclonal anti-Myc antibodies were kindly provided by Dr. Nicole Beauchemin (McGill University, Montreal, Canada).

**Expression, Purification, and Biotinylation of Recombinant Proteins**—Recombinant Rac1, Cdc42GAP-ΔGAP, Cdc42GAP-PRD, and the SH3 domains of intersectin, Nck, Src, amphiphysin I and amphiphysin II were produced in *Escherichia coli* as GST fusion proteins and purified on glutathione-Sepharose beads as described previously (23). The recombinant Rac1 was released from the beads by cleavage with human thermolin (Calbiochem). Thrombin was removed by adding 10 μl of p-aminobenzamidine-agarose beads (Sigma) for 30 min at 4 °C. Purified proteins were dialyzed against 15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.1 mM dithiothreitol, and concentrated by ultracentrifugation using the Centricron YM-10 (Amicon). GST and GST-SH3 domains were eluted from the beads with 5 mM reduced glutathione (Sigma). To biotinylate recombinant proteins, GST fusion proteins immobilized on glutathione-Sepharose beads were washed with PBS and incubated 3 h at 4 °C in the presence of Sulfo-NHS-LC-Biotin as recommended by the manufacturer (Pierce). Beads were washed extensively with PBS and proteins were eluted with 5 mM reduced glutathione (Sigma). Biotinylated proteins were immobilized on streptavidin-Sepharose beads (Pharmacia Corp.) and glutathione washed out with PBS. Protein concentration was determined using Bradford reagent (Bio-Rad) and the purity of protein preparations was visualized on Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (PAGE).

**COS-7 Cell Transfection and Pull-down Assays**—COS-7 cells were transfected by DEAE-dextran method as described previously (24). 5 μg were produced in *Escherichia coli* as GST fusion proteins and purified on glutathione-Sepharose beads as described previously (23). The recombinant Rac1 was released from the beads by cleavage with human thermolin (Calbiochem). Thrombin was removed by adding 10 μl of p-aminobenzamidine-agarose beads (Sigma) for 30 min at 4 °C. Purified proteins were dialyzed against 15 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 0.1 mM dithiothreitol, and concentrated by ultracentrifugation using the Centricron YM-10 (Amicon). GST and GST-SH3 domains were eluted from the beads with 5 mM reduced glutathione (Sigma). To biotinylate recombinant proteins, GST fusion proteins immobilized on glutathione-Sepharose beads were washed with PBS and incubated 3 h at 4 °C in the presence of Sulfo-NHS-LC-Biotin as recommended by the manufacturer (Pierce). Beads were washed extensively with PBS and proteins were eluted with 5 mM reduced glutathione (Sigma). Biotinylated proteins were immobilized on streptavidin-Sepharose beads (Pharmacia Corp.) and glutathione washed out with PBS. Protein concentration was determined using Bradford reagent (Bio-Rad) and the purity of protein preparations was visualized on Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (PAGE).

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Growth Factor Treatment and Immunofluorescence—Two hours after microinjection, Swiss 3T3 cells were stimulated with 3 ng/ml PDGF (Calbiochem) for 10 min at 37 °C and fixed for 10 min at room temperature in 4% (w/v) paraformaldehyde. All following steps were carried out at room temperature, and coverslips were rinsed in PBS between each step. Cells were permeabilized on ice for 5 min in 0.2% Triton X-100, and free aldehyde groups were reduced with 0.5 mg/ml NaBH4 for 10 min. After excellent blocking with 4% (w/v) paraformaldehyde. All following steps were carried out at room temperature, and coverslips were rinsed in PBS between each step. Cells were permeabilized on ice for 5 min in 0.2% Triton X-100, and free aldehyde groups were reduced with 0.5 mg/ml NaBH4 for 10 min.

Immunoprecipitation—COS-7 cells were transfected with pRK5-MycCdGAP together with either pRK5 or pRK5Flagintersectin by the DEAE-dextrans transfection method as described above. 48 h post-transfection, lysates of COS-7 cells overexpressing both proteins (Fig. 1A) were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin followed by centrifugation for 15 min at 10000 × g max. 1 mg of postnuclear supernatants was incubated overnight at 4 °C with 4 μg of anti-FLAG antibodies followed by a 2-h incubation with 10 μl of 50% protein G-Sepharose beads (Pharmacia Corp.). Samples were washed 3 times in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and submitted to SDS-PAGE and Western blotting analysis using anti-FLAG and anti-Myc antibodies.

In Vitro GAP Assay—COS-7 cells were transfected with pRK5, pRK5MycCdGAP, pRK5MycCdGAP-PRD, or pRK5MycCdGAP-ΔPRD by DEAE-dextran method as described above. 48 h post-transfection, Myc-tagged proteins were immunoprecipitated as described above using anti-Myc antibodies. Samples were washed 3 times in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, twice in 20 mM Tris-HCl (pH 7.5) and used for the in vitro GAP assay as following. The amount of immunoprecipitated CdGAP is estimated on Coomassie Blue-stained SDS-PAGE by comparison with different amount of purified bovine serum albumin. According to this estimation, immune complexes corresponding to 500 ng of immunoprecipitated CdGAP were resuspended in 24 μl of 20 mM Tris-HCl (pH 7.5), 0.1 mM diithiothreitol, 1 mM GTP, 0.86 mg/ml bovine serum albumin. At the same time, 2 μg of recombinant Rac1 was incubated with 5 μl of [γ-32P]GTP (30 Ci/mmol) in 20 μl of 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 0.1 mM diithiothreitol, and 5 mM EDTA for 10 min at 30 °C. GTP-loaded Rac1 was kept on ice after addition of 20 mM MgCl2, 5 μl of [γ-32P]GTP-loaded Rac1 was incubated at 20 °C with the immune complexes. After 0, 3, 6, and 9 min incubation, 4-μl mixtures were diluted in 1 ml of cold buffer A (50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2), and filtered through pre-wetted nitrocellulose filters. Filters were washed with 10 ml of cold buffer A, dried, and counted.

RESULTS

CdGAP Interacts with the SH3-containing Protein Intersectin—CdGAP consists of a N-terminal GAP domain, a central domain, and a C-terminal proline-rich domain containing five consensus SH3-binding motifs (PRD1–5) (Fig. 2A). To identify SH3 domain-containing protein(s) that bind to CdGAP, lysates of COS-7 cells expressing Myc-tagged CdGAP were incubated with SH3 domains from a variety of proteins including intersectin (SH3A-E), amphiphysin I, amphiphysin II, Nck (SH3-1–3), and Src, each expressed as GST fusion proteins and immobilized on glutathione-Sepharose beads. For direct binding, 5 μl of GST-CdGAP-GAP or GST-CdGAP-PRD were incubated with 5 μl of biotinylated GST fusion proteins immobilized on streptavidin-Sepharose beads. Samples were washed 3 times in 20 mM HEPES-OH (pH 7.4), 1% Triton X-100, submitted to SDS-PAGE, and Western blot analysis using anti-Myc or anti-CdGAP antibodies.

Cell Culture and Microinjection—Subconfluent, serum-starved Swiss 3T3 cells were prepared as described previously (10). pRK5MycCdGAP or -CdGAP-ΔPRD were microinjected at 0.04 mg/ml into the nucleus of 100 cells together with 0.16 mg/ml pRK5 or pRK5Flagintersectin. During the microinjection, cells were maintained at 37 °C with an atmosphere of 5–9% CO2. After microinjection, cells returned to the incubator for a further 2 h before treatment with extracellular factors.
only Myc-tagged CdGAP shows no CdGAP precipitation (Fig. 1B). Therefore, intersectin interacts specifically both in vitro and in cells with CdGAP through its SH3A, -B, and -D domains.

The Central Domain of CdGAP Is Required for CdGAP-Intersectin Interaction.—To characterize the interaction of CdGAP with the SH3 domains of intersectin, we generated truncated CdGAP mutants lacking either the GAP domain (CdGAP-ΔGAP) or the proline-rich domain (CdGAP-ΔPRD). CdGAP-ΔGAP corresponds to the N-terminal GAP domain whereas CdGAP-PRD consists of the proline-rich domain only (Fig. 2A). Lysates of cells expressing Myc-tagged forms of these mutant proteins were incubated with GST or GST-SH3A, -B, or -D of intersectin. Despite robust expression, both CdGAP-PRD and CdGAP-GAP mutants did not bind to GST-SH3A, -B, or -D of intersectin (Fig. 2B). In contrast, CdGAP-ΔPRD and CdGAP-ΔGAP bound to the three SH3 domains but not to GST alone, suggesting that the central domain of CdGAP is important to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro. We conclude that the CdGAP proline-rich and the N-terminal GAP domains are neither necessary nor sufficient to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro.

To determine whether intersectin-SH3 domains interact directly with CdGAP, CdGAP-ΔGAP and CdGAP-PRD were expressed as GST fusion proteins and purified on glutathione-Sepharose beads. Purified proteins were incubated with biotinylated GST, GST-SH3A, -B, or -D and immobilized on streptavidin-Sepharose beads. As shown in Fig. 3, CdGAP-ΔGAP but not CdGAP-PRD binds strongly to the SH3D and to a lesser extent to the SH3A of intersectin. No significant interaction has been observed with GST or the SH3B of intersectin. These results indicate that both SH3A and -D of intersectin interact directly with CdGAP. These data also confirm that the central domain of CdGAP is required to mediate the direct interaction between CdGAP and intersectin. Surprisingly, no consensus SH3-binding motifs are present in the central domain. Recent studies have identified non-PXXP SH3-binding domains (25, 26), however, CdGAP does not contain any of these motifs. We conclude that intersectin interacts with the central domain of CdGAP using a novel SH3-binding domain that remains to be identified.

Intersectin Co-localizes with CdGAP and Inhibits Its GAP Activity Toward Rac1 in PDGF-stimulated Swiss 3T3 Cells—Microinjection of CdGAP into serum-starved fibroblasts inhibits the Rac1-dependent formation of lamellipodia induced by PDGF (10). To determine whether intersectin regulates CdGAP activity toward Rac1 in vivo, we microinjected serum-starved, subconfluent Swiss 3T3 cells with vectors encoding intersectin, CdGAP, or both vectors. As expected, PDGF-induced membrane ruffling is abolished in ~70% of CdGAP-expressing cells (Fig. 4, A, B, and H). However, in ~80% of cells co-expressing CdGAP and intersectin, lamellipodia was formed (Fig. 4, C–H). Moreover, CdGAP and intersectin co-localize at lamellipodia (Fig. 4, E–G). Expression of intersectin alone had no effect on the actin cytoskeleton in either conditions of serum starvation or PDGF stimulation (Fig. 4, A–H; data not shown). Thus, intersectin inhibits in vivo the GAP activity of CdGAP toward Rac1 in PDGF-stimulated cells.

Intersectin Inhibits CdGAP Activity Toward Rac1 in Vitro.—To further investigate the mechanism by which intersectin inhibits CdGAP activity, we used a modified in vitro GAP assay. [γ-32P]GTP-loaded Rac1 was incubated with Myc-tagged CdGAP immobiloprecipitated from COS-7 cell lysates in the presence or absence of GST or GST-SH3D domains of intersectin. The GAPase stimulating activity was estimated by measuring the ratio of Rac1-bound radioactivity in the absence versus the presence of CdGAP using a nitrocellulose filter assay (Fig. 5A). Consistent with the previously reported GAPase stimulating activity of CdGAP toward Rac1 (10), only 10% GTP remains bound to Rac1 after a 3-min incubation with immobiloprecipitated CdGAP compared with 60% of GTP bound to Rac1 incubated with the immune complex from lysates of cells transfected with empty vector (EV). Interestingly, in the presence of GST-SH3D of intersectin, the amount of GTP remaining bound to Rac1 was increased from 10 to 45% whereas no significant effect was observed in the presence of GST or GST-SH3A or -B of intersectin. Moreover, when [γ-32P]GTP-loaded Rac1 was incubated with immobiloprecipitated CdGAP in the presence of increasing concentrations of GST fusion proteins, the inhibition of CdGAP activity was saturable and reached a plateau at 10 μM GST-SH3D (Fig. 5B). However, increasing concentrations of either GST or GST-SH3A, -B of intersectin did not affect significantly the GAPase stimulating activity of CdGAP.

When [γ-32P]GTP-loaded Rac1 was incubated with immobiloprecipitated CdGAP-PRD lacking the GAP domain, the remaining amount of GTP bound to Rac1 was comparable to the control (EV) and was not influenced by the presence of either GST or GST-SH3D (Fig. 5C). These data indicate that the proline-rich domain of CdGAP does not co-immunoprecipitate an exogenous GAP activity toward Rac1 and that intersectin-SH3D does not inhibit the intrinsic GAPase activity of Rac1. Together, these results demonstrate that the SH3D but not
SH3A or SH3B of intersectin is sufficient to inhibit specifically and directly CdGAP activity toward Rac1 in vitro in a dose-dependent manner. To better understand the in vitro inhibition of CdGAP activity by the SH3D domain only, we used competition assays to assess the possibility that intersectin-SH3D domains may interact with different sites on CdGAP. GST and GST-SH3D were biotinylated, immobilized on streptavidin-Sepharose beads, and incubated with lysates of COS-7 cells expressing Myc-tagged CdGAP and a GAP assay was performed. Incubation was carried out in the absence or presence of 10 μM GST, GST-SH3A, GST-SH3B, or GST-SH3D of intersectin. B, [γ-32P]GTP-loaded Rac1 was incubated for 3 min at 20 °C with immunoprecipitated CdGAP in the presence of 0, 5, 10, or 20 μM GST or GST fusion proteins as described in A. C, [γ-32P]GTP-loaded Rac1 was incubated for 3 min at 20 °C with anti-Myc immune protein complexes from lysates of COS-7 cells transfected with EV, pRK5/MycCdGAP-PRD (PRD), or pRK5/MycCdGAP-ΔPRD (ΔPRD). Incubation was carried out in the absence or presence of 10 μM GST or GST-SH3D. Error bars represent standard deviations relative to three separate experiments. ■, EV; □, CdGAP; △, CdGAP + GST; ▲, CdGAP + GST-SH3A; ○, CdGAP + GST-SH3B; ●, CdGAP + GST-SH3D.

results suggesting that the SH3B domain binds indirectly to CdGAP. On the other hand, the SH3A domain of intersectin is able to compete efficiently the interaction CdGAP/SH3D although at a lower extent than the SH3D domain. Since the SH3A domain was not able to inhibit the in vitro CdGAP activity, these results suggest that SH3A binds with a lower affinity to the same site within CdGAP as SH3D. However, we cannot exclude the possibility that SH3A and SH3D bind to a different site within CdGAP and that the interaction of SH3A with CdGAP may interfere with the CdGAP-SH3D interaction.

The Proline-rich Domain of CdGAP Is Required for Intersectin-mediated Regulation of CdGAP Activity Both in Vivo and in Vitro—To characterize the regulation of CdGAP activity by intersectin, vectors encoding for CdGAP-ΔPRD, intersectin, or both vectors were microinjected into serum-starved, subconfluent Swiss 3T3 cells before stimulation with PDGF. As expected, CdGAP-ΔPRD retains full enzymatic activity and inhibits PDGF-induced lamellipodia formation in ~70% of microinjected cells (Fig. 7, A, B, and H). Interestingly, intersectin was not able to inhibit the GAP activity of CdGAP missing the

Fig. 4. Intersectin inhibits CdGAP activity toward Rac1 in PDGF-stimulated Swiss 3T3 cells and co-localizes with CdGAP at the lamellipodia. Serum-starved, subconfluent Swiss 3T3 cells were microinjected with pRK5/MycCdGAP together with empty vector (A, B, and H) or pRK5/Flagintersectin (C–H) or with pRK5/Flagintersectin alone (H). 2 h later, cells were stimulated with PDGF for 10 min. CdGAP, intersectin, and actin were visualized by indirect immunofluorescence using polyclonal anti-CdGAP (CdGAP, Merge red), monoclonal anti-FLAG (intersectin, Merge green) antibodies or TRITC-coupled phalloidin (actin), respectively. Picture merge (Merge) was done using Northern Eclipse software (G). H, percentage of microinjected cells containing lamellipodia. Error bars represent the standard deviation corresponding to three separate experiments. int., intersectin. Scale bars, 5 μm.
CdGAP Regulation by Intersectin

The regulation of CdGAP activity by intersectin, a component of the endocytic machinery, interacts with CdGAP and inhibits its GAP activity toward Rac1 both in vivo and in vitro. The CdGAP proline-rich domain is absolutely required for the regulation of CdGAP activity by intersectin. However, it is neither necessary nor sufficient to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro. We demonstrate that the central domain of CdGAP is required to mediate the direct interaction between CdGAP and intersectin.

To date, the regulation of RhoGAP proteins is poorly understood. Nevertheless, the large number of RhoGAPs identified as well as their ubiquitous expression suggests that their activities may be restricted to discrete sites within the cell and/or that these activities may be tightly regulated (27). Consistent with this idea is the presence of protein- or lipid-binding modules within most proteins of the RhoGAP family. In the present study, we investigated the role of the proline-rich domain of CdGAP, which has five putative SH3-binding motifs. We show that the SH3 domain-containing protein intersectin, a major component of the endocytic machinery, interacts with CdGAP and inhibits its GAP activity toward Rac1 both in vivo and in vitro. The CdGAP proline-rich domain is absolutely required for the regulation of CdGAP activity by intersectin. However, it is neither necessary nor sufficient to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro.

**DISCUSSION**

To date, the regulation of RhoGAP proteins is poorly understood. Nevertheless, the large number of RhoGAPs identified as well as their ubiquitous expression suggests that their activities may be restricted to discrete sites within the cell and/or that these activities may be tightly regulated (27). Consistent with this idea is the presence of protein- or lipid-binding modules within most proteins of the RhoGAP family. In the present study, we investigated the role of the proline-rich domain of CdGAP, which has five putative SH3-binding motifs. We show that the SH3 domain-containing protein intersectin, a major component of the endocytic machinery, interacts with CdGAP and inhibits its GAP activity toward Rac1 both in vivo and in vitro. The CdGAP proline-rich domain is absolutely required for the regulation of CdGAP activity by intersectin. However, it is neither necessary nor sufficient to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro. We demonstrate that the central domain of CdGAP is required to mediate the direct interaction between CdGAP and intersectin. We propose that intersectin regulates CdGAP activity by inducing a conformational change involving the C terminus proline-rich domain of CdGAP (Fig. 8).

Our data demonstrate that intersectin interacts specifically with CdGAP through its SH3A, -B, and -D domains. However, only the SH3D domain was sufficient to inhibit CdGAP activity in vitro. We show that CdGAP interacts directly with SH3D and SH3A domains whereas no direct interaction with SH3B has been detected. These results may explain why, in our experimental conditions, no inhibition of CdGAP activity has been observed in the presence of the SH3B domain. Our data also suggest that SH3A may bind to the same site within CdGAP as SH3D although at a lower affinity. This may account for the inability of SH3A to inhibit CdGAP activity in vitro. However, it is also possible that SH3A and SH3D interact on different sites within CdGAP and that the interaction of SH3A with this idea is the presence of protein- or lipid-binding modules within most proteins of the RhoGAP family. In the present study, we investigated the role of the proline-rich domain of CdGAP, which has five putative SH3-binding motifs. We show that the SH3 domain-containing protein intersectin, a major component of the endocytic machinery, interacts with CdGAP and inhibits its GAP activity toward Rac1 both in vivo and in vitro. The CdGAP proline-rich domain is absolutely required for the regulation of CdGAP activity by intersectin. However, it is neither necessary nor sufficient to mediate the interaction between CdGAP and intersectin-SH3 domains in vitro. We demonstrate that the central domain of CdGAP is required to mediate the direct interaction between CdGAP and intersectin. We propose that intersectin regulates CdGAP activity by inducing a conformational change involving the C terminus proline-rich domain of CdGAP (Fig. 8).

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To further investigate the role of the proline-rich domain in the regulation of CdGAP activity by intersectin, we expressed Myc-tagged CdGAP PRD and intersectin in vitro and in vivo. Double staining for CdGAP PRD and intersectin reveals a distribution of both proteins in the perinuclear compartment as well as in small-punctuated structures in the cytoplasm (Fig. 7, E-G). We conclude that the proline-rich domain of CdGAP is required for the down-regulation of CdGAP activity by intersectin.

Protein lysates from transfected COS-7 cells expressing Myc-tagged CdGAP were incubated with 10 µg of biotinylated GST or GST-SH3D protein coupled to streptavidin-Sepharose beads. Incubation was carried out in the absence or presence of 3 or 14 µM GST-SH3A, GST-SH3B, GST-SH3D, or GST-SH3 Src. Proteins associated with biotinylated proteins were subjected to SDS-PAGE and CdGAP was detected by immunoblotting analysis using anti-Myc antibodies. The relative amount of CdGAP associated to biotinylated GST-SH3D was estimated by densitometry. Values are expressed as a percentage of SH3D-associated competition. Error bars represent standard deviations relative to two (3 µM) and three (14 µM) separate experiments.

**FIG. 6.** Intersectin-SH3A but not -SH3B competes efficiently with SH3D to interact with CdGAP. Protein lysates from COS-7 cells expressing Myc-tagged CdGAP were incubated with 10 µg of biotinylated GST or GST-SH3D protein coupled to streptavidin-Sepharose beads. Incubation was carried out in the absence or presence of 3 or 14 µM GST-SH3A, GST-SH3B, GST-SH3D, or GST-SH3 Src. Proteins associated with biotinylated proteins were submitted to SDS-PAGE and CdGAP was detected by immunoblotting analysis using anti-Myc antibodies. The relative amount of CdGAP associated to biotinylated GST-SH3D was estimated by densitometry. Values are expressed as a percentage of SH3D-associated competition. Error bars represent standard deviations relative to two (3 µM) and three (14 µM) separate experiments.

**FIG. 7.** Intersectin does not inhibit CdGAP-PRD activity toward Rac1 in PDGF-stimulated Swiss 3T3 cells. Serum-starved, subconfluent Swiss 3T3 cells were microinjected with pRK5-MycCdGAP-ΔPRD together with empty vector (A, B, and H) or pRK5Flag-intersectin (C-H) or with pRK5Flag-intersectin alone (H) followed by PDGF stimulation. CdGAP-ΔPRD, intersectin, and actin were visualized by indirect immunofluorescence using monoclonal anti-Myc (CdGAP-ΔPRD, Merge red), polyclonal anti-Flag (intersectin, Merge green) antibodies or TRITC-coupled phalloidin (Actin), respectively. Picture merge was done using Northern Eclipse software (G). H. percentage of microinjected cells containing lamellipodia. Error bars represent the standard deviation corresponding to three separate experiments. Int., intersectin. Scale bars, 5 µm.
CdGAP Regulation by Intersectin

with CdGAP interfere with CdGAP-SH3D interaction. We also show that the proline-rich domain of CdGAP is required both in vivo and in vitro for the regulation of its GAP activity by intersectin. Even though no significant binding was observed between intersectin-SH3A, -B, and -D and CdGAP-PRD mutant in vitro, in the molecular context of the full-length CdGAP protein, the proline-rich domain of CdGAP may be able to interact with intersectin. Thus, the requirement of CdGAP proline-rich domain for the regulation of CdGAP activity by intersectin may either be a consequence of a low affinity interaction between intersectin-SH3D domain and CdGAP proline-rich domain, or of a structural implication of this domain in the conformational change of CdGAP induced by intersectin. Thus, we propose a model in which the binding of intersectin-SH3D domain induces a folding of CdGAP C-terminal proline-rich domain that inhibits its GAP activity (Fig. 8).

Among all the RhoGAP proteins identified thus far, few examples of a tight regulation of their GAP activity have been described. N-chimaerin GAP activity has been shown to be directly regulated by phospholipids and phorbol ester in vitro (11, 28, 29). In contrast, p190RhoGAP activity is thought to be indirectly regulated in vivo through its interaction with p120RasGAP since this interaction stimulates its GAP activity toward RhoA in fibroblasts but not in vitro (12, 30). This indicates that p120RasGAP may regulate p190RhoGAP by targeting it to the membrane without modulating its intrinsic GAP activity. PSGAP, a novel pleckstrin homology and SH3 domain containing RhoGAP protein was shown to be regulated in vivo through its interaction with the proline-rich tyrosine-kinase 2. However, since this regulation has not been assessed in vitro (13), it is still unknown whether proline-rich tyrosine-kinase 2 directly regulates PSGAP activity or not. In the present study, we demonstrate that intersectin inhibits CdGAP activity both in vivo and in vitro, thus indicating that intersectin interaction modulates CdGAP intrinsic activity. Hence, our data provide the first demonstration of a direct regulation of RhoGAP activity mediated through a protein-protein interaction.

The endocytic scaffolding protein intersectin has been recently described as a signaling protein (14–16). It is a major partner of mSos1, a guanine exchange factor for Ras and Rac1, and is thought to play an important role in the epidermal growth factor activation of Ras (14, 15). In addition, overexpression of intersectin in fibroblasts has been shown to activate mitogenic signaling pathways (16). The regulation of CdGAP by intersectin also demonstrates the important role played by intersectin as a signaling molecule and extends its signaling properties to the regulation of Rho GTPases and actin cytoskeleton dynamics. Consistent with this latter role, the brain-specific alternative spliced variant of intersectin, named intersectin-long, contains a C-terminal extension with Dbl homology, pleckstrin homology, and C2 domains (17). Through its Dbl homology domain, intersectin-long functions as a guanine nucleotide exchange factor for Cdc42 in vitro (38). Moreover, injection of the Dbl homology domain of intersectin-long in Swiss 3T3 fibroblasts causes actin assembly leading to filopodia formation (38). Interestingly, CdGAP has a GAP activity toward Cdc42, is highly expressed in brain (10), and co-immunoprecipitates together with intersectin-long from COS-7 cells overexpressing both proteins (data not shown). Hence, the role of intersectin-long and CdGAP interaction on their respective activities toward Cdc42 is of great interest and will provide additional proof of the major role of intersectin as a scaffolding protein orchestrating Cdc42-induced signaling pathways through the recruitment and regulation of specific Cdc42 GAP, guanine nucleotide exchange factor.

In addition to its newly identified role as a signaling molecule, intersectin has been well described as a key player of the endocytic machinery (18, 20, 21, 31–33). Interestingly, several studies have highlighted a potential cross-talk between Rac1 activation and membrane trafficking, in particular endocytosis (34–37). Thus, the possible implication of CdGAP activity regulation toward Rac1 by intersectin in this cross-talk is under investigation and may provide a molecular mechanism by which the endocytic machinery may regulate Rac1 activation.

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The Activity of the GTPase-activating Protein CdGAP Is Regulated by the Endocytic Protein Intersectin

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