IQGAP1 Stimulates Actin Assembly through the N-Wasp-Arp2/3 Pathway*§

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IQGAP1 is a conserved modular protein overexpressed in cancer and involved in organizing actin and microtubules in motile processes such as adhesion, migration, and cytokinesis. A variety of proteins have been shown to interact with IQGAP1, including the small G proteins Rac1 and Cdc42, actin, calmodulin, β-catenin, the microtubule plus end-binding proteins CLIP170 (cytoplasmic linker protein) and adenomatous polyposis coli. However, the molecular mechanism by which IQGAP1 controls actin dynamics in cell motility is not understood. Quantitative co-localization analysis and down-regulation of IQGAP1 revealed that IQGAP1 controls the co-localization of N-WASP with the Arp2/3 complex in lamellipodia. Co-immunoprecipitation supports an in vivo link between IQGAP1 and N-WASP. Pull-down experiments and kinetic assays of branched actin polymerization with N-WASP and Arp2/3 complex demonstrated that the C-terminal half of IQGAP1 activates N-WASP by interacting with its BR-CRIB domain in a Cdc42-like manner, whereas the N-terminal half of IQGAP1 antagonizes this activation by association with a C-terminal region of IQGAP1. We propose that signal-induced relief of the autoinhibited fold of IQGAP1 allows activation of N-WASP to stimulate Arp2/3-dependent actin assembly. Directional cell migration results from the coordination of protrusion formation and cell adhesion. Although the concerted re-organization of actin and microtubules establishes and maintains cell polarization during directional movement, little is known about the molecular mechanisms underlying signal-mediated crosstalk between the two different cytoskeletal arrays (1). In this context, the modular IQGAP1 protein has received intense interest in the past years (2). The multiple partners of IQGAP1, including signaling molecules like Cdc42 or Rac1, calmodulin (3–6), and adhesion/cytoskeletal proteins like β-catenin, E-cadherin, actin filaments, and microtubule plus end-tracking proteins (CLIP170 and adenomatous polyposis coli (APC)) strongly suggest that IQGAP1 is an important player in coordinating cell polarity, adhesion, and migration (7–13). Concrete support to this view was brought by evidence showing that IQGAP1 is overexpressed in cancer (14, 15), controls cytokinesis (16–21), and cell-cell adhesion (22–24). In addition, recent reports showed that IQGAP1 localizes in lamellipodia of motile cells (4, 25, 26) where it may link microtubule ends to the actin cytoskeleton (12, 27) and that overexpression of IQGAP1 increases cell motility, whereas knockdown of the protein reduces cell migration and inhibits the formation of a protrusive actin meshwork at the leading edge (25). Finally, IQGAP1 regulates E-cadherin-mediated cell-cell adhesion both positively and negatively (11). However, the functional and molecular link between IQGAP1 and the actin cytoskeleton in cell-cell adhesions and in lamellipodia has remained elusive.

Extension of lamellipodia is driven by stimulus-responsive WASP family proteins (N-WASP, WASP, and Scar/WAVE) Cortactin and CARMIL, which activate the Arp2/3 complex to catalyze the formation of a branched actin filament array at the leading edge (28–34). Although WASP proteins appear to be downstream targets of Rac (35, 36), N-WASP, the ubiquitous homolog of hematopoietic WASP protein, is directly activated by Cdc42 (32, 33, 37). N-WASP is a modular protein that consists of an N-terminal WH1 region, followed by a basic region (BR), a CIB domain (Cdc42-Rac interactive binding domain), a proline-rich region, and a C-terminal catalytic domain (VCA) (Fig. 3B). N-WASP is autoinhibited by an intramolecular contact

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The abbreviations used are: BR, basic region; CRIB, Cdc42-Rac interactive binding domain; VCA, C-terminal catalytic domain; GFP, green fluorescent protein; GST, glutathione S-transferase; MDCK, Madin-Darby canine kidney cell; ROI, region of interest; siRNA, small interference RNA; GRD, Rac-GAP-related domain; GTPγS, guanosine 5′-O-(thio)triphosphate.
between the CRIB domain and the VCA domain. Cdc42-GTP and phosphatidylinositol 4,5-bisphosphate (PIP2) activate N-WASP synergistically by binding to the CRIB and the BR, respectively, to unmask the VCA domain (38, 39). The exposed VCA domain binds Arp2/3, G-actin, and an actin filament barbed end as substrates to catalyze the formation of a branched filament. The proline-rich region of N-WASP is a target for SH3 domains of a variety of adaptors like Grb2, Nck, or Abi1 (40–42) that also activate N-WASP and may act cooperatively with Cdc42, generating a graded activation response. Additionally N-WASP can be activated by direct phosphorylation, and the inhibited N-WASP-WIP complex is activated by TOCA-1 (43–46).

The detailed upstream regulation and the contribution of the different members of the WASP family proteins in cell protrusion and motility is an open issue. Here we show that the C-terminal half of IQGAP1 activates N-WASP in a Cdc42-like fashion and that this activity is abolished by an intramolecular interaction between N- and C-terminal halves suggesting that the relief of the autoinhibited fold of IQGAP1 in turn triggers unfolding and activation of N-WASP, which leads to Arp2/3-dependent actin filament branching. This mechanism emphasizes that signaling to motile events occurs through cooperative cascades of protein unfolding in membrane-associated protein complexes.

EXPERIMENTAL PROCEDURES

Antibodies—Anti N-WASP antibody was a gift from J. Peterson: NW011 (33). GFP antibodies (clones C-163 and B34) were produced at the Swiss Institute for Experimental Cancer Research antibody facility. Monoclonal and polyclonal anti-IQGAP1 were from Santa Cruz Biotechnology and BD Biosciences, respectively, anti-Arp3 was from Cytoskeleton, anti-Arp1 were from Santa Cruz Biotechnology and BD Biosciences, respectively, anti-IQGAP1 were from Santa Cruz Biotechnology and BD Biosciences, respectively, and anti-Arp3 was from Cytoskeleton, anti-actin was from Sigma, anti-GFP was from Molecular Probes, and anti-His was from Qiagen.

cDNA Constructs—Full-length GFP-N-WASP construct was a gift from P. McPherson (47). Plasmids encoding GFP fusions of the WH1, ΔWA, Δ(B-CRIB), and ΔpolyPro regions of murine N-WASP were a gift (48). The construction of pGEX4T-1-VCA (residues 392–501 of human N-WASP) was described in a previous study (33). pFastBacHTa-N-WASP was prepared by PCR using a human cDNA and the following primers:

\[\text{forward} \quad TCA\ GTC\ TTC\ CCA\ CTC\ ATC\ ATC\ ATC-3\]
\[\text{reverse} \quad \text{TCA}\ \text{GTC}\ \text{TTC}\ \text{CCA}\ \text{ATC}\ \text{ATC}\ \text{ATC}-3\]

This cDNA was cloned into pFastBacHTa vector (Invitrogen) using EcoRI/HindIII cloning sites. CRIB-BR-CRIB fragments were amplified by Pfui polymerase using primers phNW142-phNW276 and pFASTBac-NWASP as template. N-WASP BR-CRIB (amino acids 142–276) cDNA was digested with EcoRI and Xhol and cloned into pGEX4T-1. The fidelity of the constructs was confirmed by sequencing. Cloning of ΔWH1-N-WASP, ΔB-N-WASP, and N-WASP/H208D were previously described (32, 39).

Human IQGAP1-C3 was subcloned into pIJEX2.4a (Roche Applied Science), and IQGAP1-C1 was subcloned into pGEX-6P1 from clones previously described (3). Plasmid encoding His-tagged IQGAP1-N2 was a gift of G. Bloom (6). Plasmid encoding IQGAP1-C4 was a gift of K. Kaibuchi (8). Human GST-IQGAP1-C5 (amino acids 675–1657) was created by PCR (fragments 1–3: 2023–2355, 2320–4129, and 3908–4950 bp) followed by in vivo recombination in yeast. Briefly, PCR fragments, including the restriction sites Xmal/SacII/Xhol and XbaI, were co-transformed into yeast together with a BamHI linearized yeast shuttle vector pCJ36.7 The resulting vector was used for re-cloning IQGAP1-C5 into pGEX-6P1. The fidelity of the construct was confirmed by sequencing.

Cell Culture and Immunofluorescence—MDCK cells were seeded on coverslips and grown in minimal essential medium (Sigma) containing 10% fetal calf serum (Cambrex), 1 μg/ml penicillin/streptomycin, and 2 μM glutamine (Invitrogen). After 24 h cells were starved in medium without fetal calf serum. 16 h later cells were stimulated with minimal essential medium containing 10% fetal calf serum for 10 min, fixed at 37 °C, and stained with primary antibodies against Arp3, IQGAP1, and N-WASP followed by secondary antibodies conjugated with Coumarin Phalloidin, Alexa488, Alexa594, or Alexa647 (Molecular Probes, Invitrogen). Co-localization data originate from multichannel fluorescence XY stacks of islands containing 2–6 cells, which were collected using a confocal laser scanning microscope (Leica TCP-SP2 AOBs, Leica Microsystems). All images were recorded with a high resolution oil immersion objective (PlanApo, 63×, numerical aperture 1.4, Leica) tuning the sampling density to meet the requirements of the Nyquist theorem.

In two-color specimens, Alexa488 fluorescence was excited with the 488 nm line of an argon laser and collected in the 495–580 nm optical window, whereas, in parallel, Alexa594 fluorescence was excited with the 594 nm line of an HeNe laser and collected in the 605–770 nm optical window.

In three-color specimens serial intra-frame acquisition of the three fluorescence channels was necessary to minimize crosstalk between Alexa594 and Alexa647 fluorescence emissions. First, Alexa488 fluorescence was excited with the 488 nm line of an argon laser and collected in the 495–580 nm optical window, whereas, in parallel, Alexa594 fluorescence was excited with the 594 nm line of an HeNe laser and collected in the 605–770 nm optical window. Second, Alexa594 fluorescence was excited with the 561 nm line of a HeNe laser and collected in the 605–770 nm optical window.

Crosstalk between fluorescence channels was assessed in one-color specimens, and the best optical settings were designed with the help of the Fluorescence Spectra Viewer (Molecular Probes) to reduce crosstalk values to <5% maximum intensity.

In all specimens an additional transmission channel was recorded in parallel to visualize the two-dimensional profile of the cells under study. Laser intensities and channel gains were tuned to minimize photobleaching and to obtain similar signal to noise ratios in the fluorescence channels.

Image Processing and Co-localization Measurement—Image processing and deconvolution were done with the Huygens software package (Scientific Volume Imaging) imposing the optical parameters used to collect the fluorescence XY stacks.

7 Y. Barral, unpublished data.
Deconvolution was necessary to remove background and other optical artifacts (e.g., chromatic aberration) that can introduce false positive or false negative voxels in the co-localization analysis (49). Deconvolved XY stacks were loaded in the Huygens essential version 7.1 and analyzed as follows. An individual cell in the cell island was cropped in three-dimensions using the “crop” tool of Huygens. A region of interest (ROI) was then defined using the “object analyzer tool” of Huygens and than exported to the XY stack under analysis. A careful definition of the ROI was obtained, for each cell in the island, manually outlining on the two-dimensional projection a 3-μm thick closed line from the edge toward the cell cytoplasm. The extracellular voxels selected above and below the cell in the ROI drawn were removed using the crop tool of Huygens to obtain a refined ROI. The cellular voxels selected in this way, containing only the fluorescence signal from the cell’s leading edge, represented the functional co-localization of the proteins under study and thus needed to be measured.

The channel co-localization in these voxels was measured using the “co-localization analyzer tool” of Huygens. For each cell a single value of the Pearson’s coefficient in the refined ROI was measured imposing a threshold value for both channels. The threshold was calculated on both channels based on an algorithm implemented in the Imaris software package (Bitplane) and obtained using the “automatic thresholding” function of Imaris. Around 20 cells from islands of 2–6 cells were measured per condition.

Unspecific co-localization was calculated for each deconvolved XY stack selecting an ROI outside the cell island containing appreciatively the same number of voxels as the cellular ROI and measuring the relative Pearson’s coefficient as described above. This value accounted for residual crosstalk between the two channels and for unspecific antibody clustering in the specimen and was back-subtracted from the Pearson’s coefficients measured in the cellular ROIs.

siRNA—RNA oligonucleotides containing 21 nucleotides in sense and antisense direction were designed according to dog IQGAP1 (accession number: XM_536199). SiIQGAP1 (sense sequence: UGAAGCCAUUGACCAUAGAdTdT) and the Scramble oligonucleotide (sense sequence: UUCUCCGAACGUGUCACGudTdT) were from Qiagen. A second IQGAP1 specific siRNA oligonucleotide was used, and comparable results were obtained. MDCK cells were transfected twice using...
HiPerfect transfection reagent (Qiagen) according to the manufacturer’s protocol.

**Statistical Analysis**—Statistical comparisons of Pearson’s coefficients measured in individual cells from two to three independent specimens were performed with a one-tailed two-sample *t* test (*α* = 0.05) for all data sets (Figs. 1E, 1F, 1G, and 2B) using the “Two Sample *t*-Test” function of the software OriginPro. Normal distribution of the data sets could be assessed with a Shapiro-Wilk test (*α* = 0.05) using the “Normality Test” function of OriginPro. Statistical significance was further verified using the more stringent two-tailed non-parametric Mann-Whitney test. This test (*α* = 0.05) on data sets presented in Fig. 1 (E–G) confirmed a significantly increased co-localization upon serum stimulation between IQGAP1/N-WASP (*p* < 0.0001), between Arp3 and IQGAP1 (*p* < 0.0001), and between Arp3 and N-WASP (*p* = 0.002). Two-tailed Mann-Whitney tests (*α* = 0.05) on data sets presented in Fig. 2B confirmed a significantly decreased co-localization between Arp3 and N-WASP in siIQGAP1-treated cells (*p* = 0.0078).

**Proteins**—Actin and Arp2/3 were purified from rabbit muscle and bovine brain (33), respectively. His-IQGAP1-N2, -C3, and GST-tagged IQGAP1-C1, -C4, -C5, and -N2 were expressed in *Escherichia coli* strain BL21(DE3)pLyS5 and induced with 0.1 mm isopropyl-1-thio-β-d-galactopyranoside. The cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM MgCl2), sonicated, and centrifuged for 30 min at 11,000 rpm. All affinity purification steps were done according to the bead manufacturer’s instructions. Various fragments of IQGAP1 and N-WASP were expressed in *E. coli* as GST fusions using pGEX-6P-1 and purified on glutathione-Sepharose beads. GST was cleaved off using PreScission protease (Amersham Biosciences). His-tagged proteins were purified using nickel-nitritoriacetic acid beads (Qiagen). GST-VCA was thrombin-cleaved (Sigma).

**Immunoprecipitation**—HEK 293 cells were transfected with various GFP-tagged proteins using Effectene. Cells were lysed in NETN buffer (20 mM Tris-HCl, pH 7.5, at 4 °C, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1.4 μg/ml aprotinin, 10 mM sodium vanadate, 40 mM β-glycerol phosphate, 1 mM benzamidine, 1 μM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride, 1 mM dithiothreitol) on ice for 5 min. Cells were scraped and pelleted. 40 μl of the supernatant was used for detection of transfected proteins, 250 μl was used to immunoprecipitate the GFP-tagged proteins (anti-GFP antibody, Molecular Probes), and the rest was used to immunoprecipitate endogenous IQGAP1 (anti-IQGAP1 antibody) overnight. Afterward 20 μl of NETN buffer equilibrated protein A-Sepharose was added, and rotation was continued for another 3 h. Protein A-Sepharose was pelleted, the supernatant was aspirated, and beads were washed in NETN buffer. Protein A-Sepharose was boiled in SDS-loading buffer and separated by SDS-PAGE, followed by immunoblotting.

**Actin Polymerization Fluorescent Assay**—Actin polymerization was monitored by the increase in fluorescence of 10% pyrenyl-labeled actin. Actin polymerization was induced by addition of 100 mM KCl, 1 mM MgCl2, and 0.2 mM EGTA to a solution of Ca-ATP-G-actin in G buffer (5 mM Tris-Cl, pH 7.8, 0.1 mM CaCl2, 0.2 mM ATP, 1 mM dithiothreitol) containing the desired proteins. Fluorescence measurements were carried out in a Safas spectrofluorometer. Maximal rates of polymerization were measured at half-polymerization time. Relative activation of N-WASP by IQGAP1, *R*, was calculated as follows: 

\[ R = \frac{V_x - V_{min}}{V_{max} - V_{min}} \]

in which *V* <sub>x</sub> is the maximal rate of polymerization in the presence of a given concentration of IQGAP1 and 87 nm N-WASP, *V* <sub>min</sub> is the maximal rate of polymerization in the absence of IQGAP1 and 87 nm N-WASP, and *V* <sub>max</sub> is the maximal rate of polymerization at saturation of 87 nm N-WASP by IQGAP1.

**Observation of Branched Actin Filaments**—Actin (2.5 μM) was polymerized in the presence of 25 nm Arp2/3 and 175 nm N-WASP in the absence or presence of 600 nm IQGAP1-C4. As soon as 90% polymerization was reached actin filaments were stabilized and stained with a molar equivalent of Alexa488.
Phalloidin, diluted 120-fold in 5 mM Tris-Cl/H11002, pH 7.8, 100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 0.2 mM ATP, 10 mM diethiothreitol, 0.1 mM 1,4-diazabicyclo[2.2.2]octane, and 0.1% methylcellulose and processed for observation in an Olympus AX-70 microscope with a 100× objective and a Cascade II video camera (Photometrics). The density of branching was defined as the number of branches formed per unit length of filaments, counting a total length of at least 5000 μm. The length was measured using a Metamorph software while branches were counted manually. The experiment was reproduced three times with the same result.

RESULTS

IQGAP1 Co-localizes with an Actin Polymerization Machinery in Newly Formed Lamellipodia—Serum stimulation of living cells activates a plethora of signaling pathways leading to site-directed actin assembly that drives membrane ruffling and lamellipodia extension. We observed that newly polymerized actin co-localized with Arp3 and IQGAP1 (Fig. 1A). Therefore, we analyzed the level of co-localization of IQGAP1, N-WASP, and Arp2/3 in lamellipodia (Fig. 1, B–G). In starved cells, IQGAP1, N-WASP, and Arp3 displayed punctuate staining and little accumulation in close proximity to the periphery of a protrusion. Upon serum stimulation, all three proteins accumulated in line-like structures (1–2 μm thick) in close proximity to the plasma membrane, thus changing qualitatively and quantitatively their relative positions. Serum stimulation increased the co-localization by 67.7 ± 8.7% between IQGAP1 and N-WASP, by 83.7 ± 10.5% between IQGAP1-Arp3 and by 26.6 ± 2% between N-WASP and Arp3 (Fig. 1, E–G). The increase of >2-fold in the co-localization index between IQGAP1/N-WASP or IQGAP1/Arp3 compared with N-WASP/Arp3 suggests a functional link between IQGAP1 and the N-WASP-Arp2/3 machinery.

IQGAP1 Regulates the Co-localization of Arp2/3 and N-WASP—It was previously reported that the depletion of IQGAP1 using siRNA resulted in the loss of lamellipodia (12). To evaluate whether IQGAP1 controls the localization of N-WASP and Arp3 in lamellipodia we found conditions in which IQGAP1 expression was specifically but only partially down-regulated (to 20%) using siRNA to allow the formation of lamellipodia (Fig. 2, A and C). The analysis of IQGAP1 down-regulated cells revealed quantitatively a significant reduction in the co-localization of N-WASP/Arp3 compared with N-WASP/Arp3 in lamellipodia (12). To evaluate whether IQGAP1 controls the localization of N-WASP and Arp2/3 in lamellipodia we found conditions in which IQGAP1 expression was specifically but only partially down-regulated (to 20%) using siRNA to allow the formation of lamellipodia (Fig. 2, A and C). The analysis of IQGAP1 down-regulated cells revealed quantitatively a significant reduction in the co-localization of N-WASP and Arp3 in the analyzed volume of the protrusions of stimulated cells compared with the control (Fig. 2, B and C). The reduction in the Pearson coeffi-

FIGURE 3. IQGAP1 associates with N-WASP in vivo. A, diagram of IQGAP1 constructs. B, diagram of N-WASP constructs. C, lysates of HEK293 cells, overexpressing various GFP-labeled N-WASP variants, were immunoprecipitated (IP) with anti-IQGAP1 antibodies (left panels) and anti-GFP antibodies for input control, respectively (right panel). Immunoprecipitates were blotted with indicated antibodies (WB). Arrowheads indicate the position of the respective GFP or GFP-N-WASP fragments.
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Interaction between IQGAP1 and N-WASP—The in vivo interaction between IQGAP1 and N-WASP was further analyzed by co-immunoprecipitation (Fig. 3C). Various GFP-tagged fragments of N-WASP (Fig. 3B) were overexpressed, and endogenous IQGAP1 was immunoprecipitated. Full-length IQGAP1 bound full-length N-WASP, the WH1 region, as well as N-WASP devoid of either the VCA domain or the proline-rich region. However, the interaction was almost undetectable when BR-CRIB (A(B-CRIB)) was deleted, suggesting that in vivo IQGAP1 interacts, directly or indirectly, with the BR-CRIB and WH1 regions of N-WASP (Fig. 3C).

The C-terminal Half of IQGAP1 Induces N-WASP-mediated Actin Polymerization—To directly analyze the function of IQGAP1 on actin polymerization, different bacterial recombinant fragments were produced (Fig. 3A). C-terminal fragments spanned the GRD (Ras-GAP-related domain) (C1), the IQ, GRD, and C-terminal domain (C4), and the WW, IQ, GRD, and C-terminal domain (C5), and an N-terminal fragment encompassing the CH domain and the coiled-coil domain (N2). We used a fluorescence pyrenyl-actin polymerization assay to evaluate the effect of IQGAP1 fragments on the ability of N-WASP to activate the Arp2/3 complex. The IQGAP1 fragments C4, C5, and C1 stimulated actin assembly in the presence of N-WASP and Arp2/3 but did not affect polymerization of actin in the presence of Arp2/3 alone, demonstrating that IQGAP1 binds to and activates N-WASP (Fig. 4A). Half-maximal stimulation of filament assembly was observed at 15 nM for C4 or C5 and 110 nM for C1, respectively, which is comparable to other N-WASP activators (33, 40, 41) (Figs. 4B and S1).

Quantitative analysis of the polymerization kinetics indicated that the filament barbed end concentration was increased by C4 from 0.3 nM to 0.8 nM (Fig. 5A). It is well established that N-WASP and Arp2/3 nucleate new filaments by branching existing filaments in an autocatalytic reaction (31). The direct observation and quantification of branch spacing of actin filament fragments showed that C4 induced a 3-fold increase in filament barbed ends obtained by time derivation of the polymerization kinetics presented in supplemental Fig. S1 at the indicated concentrations of IQGAP1-C4 in the presence of 67 nM Arp2/3 and 87 nM N-WASP (31). Inset: the maximum concentration of filament is plotted versus the concentration of IQGAP1-C4. B, IQGAP1 increases the frequency of actin filament branching. Actin (2.5 μM) was polymerized in the presence of 25 nM Arp2/3 and 175 nM N-WASP in the absence (left) or presence (right) of 600 nM IQGAP1-C4, stained with 2.5 μM Alexa488-Phalloidin and diluted 120-fold before observation in a fluorescent microscope. Bar = 5 μm. C, the frequency of branching (branches/μm of actin filament) was calculated from the experiment described in Fig. S5B. Data shown are average ± S.E.
Diated filament branching. Interestingly, C4 failed to activate WAVE-1 showing a high specificity of IQGAP1 for N-WASP among the WASP family proteins (Fig. 6).

**IQGAP1 Relieves the Autoinhibition of N-WASP.** To go further into the mechanism of activation of N-WASP by IQGAP1 we compared the ability of IQGAP1 to activate either wild-type, mutated N-WASP or the constitutively active VCA domain of N-WASP. N-WASP was still activated by C4 or C5 following deletion of the WH1 domain, indicating that the interaction of WH1 with IQGAP1 observed in vivo is either indirect or does not affect N-WASP activity (Fig. 7A). On the other hand, deletion of the BR abolished the activation by both C4 and C5, whereas it did not affect the activation by Cdc42 (Fig. 7A, inset). Neither C4 nor C5 were able to activate the VCA domain of N-WASP (Fig. 7A). Hence the BR was required for binding and activation of N-WASP by IQGAP1, consistent with the significance of this region for the in vivo interaction with IQGAP1 (Fig. 3C).

To elucidate whether IQGAP1 could activate N-WASP by relieving the autoinhibitory interaction in a Cdc42-like fashion, we used the recombinant BR-CRIB fragment that contains both the putative IQGAP1 binding domain (BR) and the autoinhibitory region of N-WASP (CRIB). Pull-down assays demonstrated that C4 binds to BR-CRIB of N-WASP as well (Fig. 7B, data not shown). In addition, C4 relieved the inhibition of VCA by BR-CRIB in a dose-dependent fashion in actin polymerization assays, demonstrating that C4 not only binds to the BR-CRIB of N-WASP with high affinity but also disrupts the CRIB-VCA interaction (Fig. 7C). Interestingly, we identified the position of the binding domain of IQGAP1 very close to the Cdc42 binding domain (CRIB) raising the question of a possible synergistic activation of N-WASP by IQGAP1 and Cdc42. In the presence of a saturating concentration

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**FIGURE 6.** IQGAP1-C4 does not activate WAVE-1. IQGAP1-C4 (312 nM) stimulates actin assembly in the presence of 60 nM Arp2/3 and 43 nM N-WASP but had no effect in the presence of 60 nM Arp2/3 and 60 nM or 120 nM WAVE-1.

**FIGURE 7.** The C-terminal half of IQGAP1 activates N-WASP by binding to its BR to disrupt the autoinhibitory interaction. A, IQGAP1-C4 and IQGAP1-C5 (312 nM) activate N-WASP lacking the WH1 domain (∆WH1-N-WASP) (47 nM) but have no effect on N-WASP lacking the BR (∆B-N-WASP) (47 nM) nor on the VCA domain (47 nM). Inset, ∆B-N-WASP is activated by Cdc42-GTP·γS. Actin assembly is measured in the presence of 12.5 nM Arp2/3 (black) and 107 nM ∆B-N-WASP without (thin red) and with 2.5 μM Cdc42-GTP·γS (thick red). B, GST-IQGAP-C4 binds directly to BR-CRIB of N-WASP. GST-tagged IQGAP1-C4 (10 μM) or GST (10 μM) immobilized on 20 μl of glutathione-Sepharose 4B were mixed with BR-CRIB (2 μM) in 200 μl of buffer B (20 mM Tris-Cl, pH 7.8, 100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 0.05% Tween 20) and incubated for 2 h at 4 °C on a rotating wheel. Beads were washed three times with 200 μl of buffer B. Total input (T) of BR-CRIB, bound fractions (P), and supernatants (S) (non-diluted) were submitted to SDS-PAGE, and the BR-CRIB domain was detected by Western blotting using a polyclonal anti-N-WASP antibody, whereas GST and GST-C4 were revealed by Coomassie Blue staining. C, increasing concentrations of C4 reverse the inhibition of VCA (75 nM) by the BR-CRIB domain (156 nM).
of Cdc42, addition of a saturating concentration of C4 further activated N-WASP (Fig. 8, A and B). To rule out a possible direct effect of Cdc42 on C4 we used a mutant of N-WASP/H208D that does not bind Cdc42 (32). We found that this mutant is activated by C4, but the addition of Cdc42 had no effect (Fig. S2). Altogether these data showed that Cdc42 and IQGAP1 bind simultaneously to the same N-WASP molecule and activate it synergistically.

IQGAP1 Is Autoinhibited—The fact that IQGAP1 was recruited into a specific line structure at the leading edge in response to extracellular stimuli (Figs. 1 and 2) suggests that the activity of the C-terminal domain must be regulated in the full-length protein. To address this issue, pull-down assays were used to evaluate the interaction between a C-terminal and a N-terminal part of IQGAP1 (Fig. 9A). We found that N2 (1875 nM) reverses the activation of N-WASP (175 nM) by C5 (600 nM) in the presence of 25 nM Arp2/3 but has no effect on N-WASP. C, the maximum rate of actin assembly is plotted as a function of the concentration of N2 in the presence of 175 nM N-WASP, 25 nM Arp2/3, 300 nM C5 (left), or 300 nM C4 (middle) or 1000 nM GST-C1 (right).
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FIGURE 10. Model for the molecular mechanism by which IQGAP1 promotes actin assembly. In non-stimulated cells IQGAP1 is in an autoinhibited fold, in which the activating C-terminal domain (green) is sequestered by the inhibiting N-terminal domain (red). Stimulation of IQGAP1 by activators could lead to the removal of the N-terminal inhibition and unfolding of the C-terminal half. In its activated state the C-terminal domain of IQGAP1 interacts with the BR-CRII region (B-C) of N-WASP to unmask VCA that binds Arp2/3 (2/3). Activation of Arp2/3 induces the formation of branched actin filaments necessary for the extension of a lamellipodium.

DISCUSSION

Lamellipodium Extension Is Promoted by N-WASP upon Activation by IQGAP1—Extension of lamellipodia and filopodia is mediated by the maintenance of dynamic meshworks of actin filaments. N-WASP and WAVE proteins, which use Arp2/3 complex to promote the formation of densely branched filaments, are present and functional at the leading edge of lamellipodia but are activated by different pathways, probably corresponding to the different cellular functions in which IQGAP1 is involved like cell-cell adhesion and lamellipodium extension. The present results bring some light in this issue in showing that IQGAP1 directly binds and remodels actin filaments (7), suggesting that IQGAP1 has the ability to remodel the actin network in different ways corresponding to the different cellular functions in which IQGAP1 is involved like cell-cell adhesion and lamellipodium extension.

IQGAP1 adds to the large number of known activators of N-WASP, including Cdc42, phosphatidylinositol 4,5-bisphosphate, Grb2, Nck, Abi1, and Endophilin A (32, 40–42, 51), that specify the localization and cell function in which N-WASP is activated. Here we show that IQGAP1 stimulates N-WASP in the lamellipodium, because the RNA interference depletion of IQGAP1 leads to a decrease in N-WASP and Arp2/3 recruitment in dynamic lamellipodia.

In living cells, N-WASP is likely activated by a cooperative mechanism in which combinations of regulators are used to integrate and amplify signals. Here we confirm this view by showing that Cdc42 and IQGAP1, two regulators of cell migration, activate N-WASP synergistically.

Mechanism of Activation of N-WASP by IQGAP1—The C-terminal region of IQGAP1 activates N-WASP, the isolated C-terminal and N-terminal domains of IQGAP1 interact with each other, and the N-terminal domain abolishes the activation of N-WASP by the C-terminal domain. These facts strongly suggest that in quiescent cells full-length IQGAP1, like its target N-WASP, adopts an autoinhibited fold (Fig. 10). Cell stimulation initiates a cascade of events. We previously showed that phosphorylation of serine 1443 opens the structure of the C-terminal half of IQGAP1 (3). It is thus possible that phosphorylation of serine 1443 relieves the autoinhibited fold, allowing the exposed C-terminal half to activate N-WASP. The C-terminal domain would then interact with the BR-CRII region of N-WASP, thus unmasking the VCA domain, which in turn interacts with Arp2/3 to promote the formation of a branched filament array (38, 42, 52).

Perspectives—The nature of input signals that activate IQGAP1 in IQGAP1-dependent cell processes is an open issue. IQGAP1 plays an important role in adherent junctions by binding to E-cadherin and β-catenin (23). Recent studies reveal that the activation of Arp2/3 is involved in nascent and mature junctions (53, 54). The present work provides the molecular tools to understand whether and how IQGAP1 controls actin assembly in cadherin-mediated cell-cell adhesion.

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