IQGAP1 Promotes Neurite Outgrowth in a Phosphorylation-dependent Manner*

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In eukaryotic cells IQGAP1 binds to and alters the function of several proteins, including actin, E-cadherin, β-catenin, Cdc42, and Rac1. Yeast IQGAP homologues have an important role in cytoskeletal organization, suggesting that modulation of the cytoskeleton is a fundamental role of IQGAP1. Phosphorylation is a common mechanism by which cells regulate protein function. Here we demonstrate that endogenous IQGAP1 is highly phosphorylated in MCF-7 human breast epithelial cells. Moreover, incubation of cells with phorbol 12-myristate 13-acetate (PMA) stimulated phosphate incorporation into IQGAP1. By using mass spectrometry, Ser-1443 was identified as the major site phosphorylated on IQGAP1 in intact cells treated with PMA. Ser-1441 was also phosphorylated but to a lesser extent. In vitro analysis with purified proteins documented that IQGAP1 is a substrate for protein kinase C, which catalyzes phosphorylation on Ser-1443. Consistent with these findings, inhibition of cellular protein kinase C via bisindolylmaleimide abrogated Ser-1443 phosphorylation in response to PMA. To elucidate the biological sequelae of phosphorylation, Ser-1441 and Ser-1443 were converted either to alanine, to create a nonphosphorylatable construct, or to glutamic acid and aspartic acid, respectively, to generate a phosphomimetic IQGAP1. Although overexpression of wild type IQGAP1 enhanced neurite outgrowth in N1E-115 neuroblastoma cells, the nonphosphorylatable IQGAP1 S1441A/S1443A had no effect. In contrast, the S1441E/S1443D mutation markedly enhanced the ability of IQGAP1 to induce neurite outgrowth. Our data disclose that IQGAP1 is phosphorylated at multiple sites in intact cells and that phosphorylation of IQGAP1 will alter its ability to regulate the cytoskeleton of neuronal cells.

Initially identified 10 years ago, IQGAP1 has been shown to participate in several fundamental cellular processes (for reviews see Refs 1 and 2). These include cell-cell attachment, β-catenin-mediated transcription, cell migration, regulation of actin, microtubule function, the mitogen-activated protein kinase cascade, and Ca2+/calmodulin signaling (2–4). IQGAP1 is a component of these diverse functions via direct interactions with multiple target proteins that are mediated by a number of protein interaction motifs in IQGAP1. These include the following: a calponin homology domain, responsible for actin binding (5, 6); a WW motif, which is necessary for the association of extracellular signal-regulated kinase 2 (a component of the mitogen-activated protein kinase pathway) (4); a calmodulin-binding IQ domain (5, 7); and a RasGAP-related domain that binds the small GTPases Cdc42 and Rac1 (5, 8). In addition, IQGAP1 binds to and regulates the functions of E-cadherin, β-catenin, and CLIP-170 (9–12).

Accumulating evidence reveals an important role for IQGAP1 in cytoskeletal function. The cytoskeleton of eukaryotic cells comprises several elements, including actin, microtubules, and intermediate filaments. Numerous proteins interact with the actin cytoskeleton and regulate its function. Major regulatory proteins include the Rho family GTPases (Rho, Rac, and Cdc42), myosin, the Ena/VASP family, Arp 2/3, profilin, β-catenin, and IQGAP1 (13, 14). Yeast IQGAP homologues participate in the recruitment of actin filaments and are required for actomyosin ring assembly (2). Mammalian IQGAP1 enhances actin polymerization in vitro (15, 16) and colocalizes with actin in lamellipodia (8). In addition, IQGAP1 regulates the actin cytoskeleton indirectly via Cdc42 and Rac1 (17) and captures growing microtubules via CLIP-170 (12).

Phosphorylation is a major post-translational method for regulating protein function, including that of the cytoskeleton. The important role of phosphorylation as a component of the changes in the cytoskeleton produced by malignant transformation has been known for some time (18). More recent evidence reveals that phosphorylation is essential for Ena/VASP function in cell movement (19), and the activity of Rho is regulated by tyrosine phosphorylation of RhoGEFs (20). Moreover, phorbol esters modulate cell motility via Rac, Cdc42, and ezrin (21–23), and Hek-induced tyrosine phosphorylation of Wiskott-Aldrich syndrome protein results in filopodia (24). In this work we demonstrate that endogenous IQGAP1 is highly phosphorylated in cells and that IQGAP1 is a target for protein kinase C (PKC). The specific phosphorylation sites were identified by mass spectrometry, and point mutant constructs of IQGAP1

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1 The abbreviations used are: PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene difluoride; EGF, enhanced green fluorescent protein; GST, glutathione-S-transferase; MAP-2, microtubule-associated protein 2; TRITC, tetramethylrhodamine isothiocyanate; HPLC, high pressure liquid chromatography; LC-ESMS, liquid chromatography electrospray mass spectrometry.
were generated. A phosphomimetic mutant of IQGAP1 dramatically enhanced its ability to promote neurite outgrowth, suggesting that IQGAP1 regulates the neuronal cytoskeleton in a phosphorylation-dependent manner.

### EXPERIMENTAL PROCEDURES

**Materials**—PKC and PKCe were purchased from Promega and Panvera, respectively. Polyvinylidene difluoride (PVDF) was from Millipore. Tissue culture reagents and fetal bovine serum (FBS) were obtained from Invitrogen. Anti-Myc monoclonal antibodies (9E10.2) were manufactured by Maine Biotechnology. The anti-IQGAP1 polyclonal antibody has been characterized previously (5). Secondary antibodies for ECL detection were from Amersham Biosciences. Bisindolylmaleimide was purchased from Calbiochem. 32P- and [γ-32P]ATP were from PerkinElmer Life Sciences. All other reagents were of standard analytical grade.

**IQGAP1 Plasmid Construction**—Myc-tagged wild type IQGAP1 (amino acids 2–1657), IQGAP1-N (amino acids 2–863), and IQGAP1-C (amino acids 864–1657) in the pcDNA3 vector have been described previously (5). To perform site-directed mutagenesis, the C-terminal half of IQGAP1 was inserted into pBluescript KS to produce pBluescript-IQC. Site-directed mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene). IQGAP1 S1441A/S1443A (amino acids 1441 and 1443, Ser to Ala) was generated using 5′-CCCTGACAAGATTGAAAAAGCAGAATTG-GAAGAAG-3′ and 5′-CTCCCTTTACACCGTCCTTTATCCCCG-3′ (mutated residues are in boldface and underlined). For IQGAP1 S1441E/S1443D (amino acids 1441 and 1443, Ser to Glu and Ser to Asp, respectively), the primers 5′-CCCTGACAAGATTGAAAAAGCAGAATTG-GAAGAAG-3′ and 5′-CTCCCTTTACACCGTCCTTTATCCCCG-3′ were used. After mutagenesis, the ClaI-XbaI fragment of pBluescript-IQC was re-inserted into pcDNA3 from which the wild type IQGAP1 C-terminal region had been removed. The sequence of all constructs was confirmed by DNA sequencing. Plasmids were purified with a QiaPrep Spin Miniprep kit (Qiagen) according to the manufacturer’s instructions.

IQGAP1 constructs were tagged with tandem enhanced green fluorescent protein (EGFP) by digesting IQGAP1 in pcDNA3 with XbaI. After making a blunt end with T4 polymerase, partial digestion was performed with BamHI. The 5577-bp fragment was inserted into the pcDNA3 vector. After making a blunt end with T4 polymerase, partial digestion was performed with BamHI. The 5577-bp fragment was inserted into pcDNA3. A phosphomimetic mutant of IQGAP1 dramatically enhanced its ability to promote neurite outgrowth, suggesting that IQGAP1 regulates the neuronal cytoskeleton in a phosphorylation-dependent manner.

**IQGAP1 Induces Neurite Outgrowth**

In vitro with pure PKC. GST-IQGAP1 was expressed in E. coli and isolated with glutathione-Sepharose as described previously (5). Endogenous IQGAP1 was immunoprecipitated from serum-starved, unstimulated MCF-7 cells as described above, except cells were not incubated with 32P. Samples on glutathione-Sepharose (GST-IQGAP1) or protein A-Sepharose (endogenous IQGAP1) beads were washed twice in PKC assay buffer (20 mM Hepes, pH 7.4, 157 mM NaCl, 10 mM KCl, and 1 mM dithiothreitol), and beads were resuspended in 10 μl of PKC buffer containing 8 μl of 6.25 mg/ml phosphatidylserine. After adding 1 μl of [γ-32P]ATP (10 μCi), the reaction was initiated with 1 μl of PKC. Samples were incubated at 30 °C for 5 min with gentle agitation every 1 min. The beads were pelleted by centrifugation, washed, and resuspended in SDS-PAGE solubilization buffer. SDS-PAGE was performed, followed by transfer to antibody and autoradiography.

In vitro with PKC for PKCe—Phosphorylation of IQGAP1 purified from cells and GST-IQGAP1 was performed in vitro with pure PKC. GST-IQGAP1 was expressed in E. coli and isolated with glutathione-Sepharose as described previously (5). Endogenous IQGAP1 was immunoprecipitated from serum-starved, unstimulated MCF-7 cells as described above, except cells were not incubated with 32P. Samples on glutathione-Sepharose (GST-IQGAP1) or protein A-Sepharose (endogenous IQGAP1) beads were washed twice in PKC assay buffer (20 mM Hepes, pH 7.4, 157 mM NaCl, 10 mM KCl, and 1 mM dithiothreitol), and beads were resuspended in 10 μl of PKC buffer containing 8 μl of 6.25 mg/ml phosphatidylserine. After adding 1 μl of [γ-32P]ATP (10 μCi), the reaction was initiated with 1 μl of PKC. Samples were incubated at 30 °C for 5 min with gentle agitation every 1 min. The beads were pelleted by centrifugation, washed, and resuspended in SDS-PAGE solubilization buffer. SDS-PAGE was performed, followed by transfer to antibody and autoradiography.

**Phosphorylation Site Analysis of IQGAP1**—Phosphopeptide-specific LC-ESMS was performed as described (26). Tryptic peptides from IQGAP1 were separated by capillary reverse phase HPLC using acetone/water gradients flowing at 4 μl/min. Negative ion LC-ESMS using single ion monitoring for the phosphate-specific marker ions m/z 63 (PO3−) and m/z 80 (PO4−) was performed as described previously (27) on a PE-Sciex API III+ (Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Micromass nanoflow ion source as described above. The HPLC eluent was split post-column with 0.4–0.6 μl/min going to the mass spectrometer and the remainder being sent to a preparation line for manual fraction collection into PCR tubes. Fractions were immediately stored at −70 °C until analyzed.

**Precursor ion scanning for m/z 79** was performed on a Sciex API 3000 triple quadrupole mass spectrometer equipped with a nanoelectrospray source. One-half of each HPLC fraction collected in the experiment described above was made basic by adding 2 volumes of 50/50 methanol/water containing 10% ammonium hydroxide (30 weight %), and 1.5 μl was loaded into the nano needle for analysis. Spectra were acquired in the multi-channel analyzer mode as described previously (28). Alternatively, precursor ion scanning for m/z 79 was performed online during LC-ESMS using a Sciex API 4000 triple quadrupole-linear ion trap (29). Tryptic peptides from IQGAP1 were separated by nanobore (75 μm inner diameter) reversed phase HPLC using acetonitrile/water gradients flowing at 0.3 μl/min. A makeup flow of 0.3 μl/min acetonitrile was added post-column by means of a tee. The entire HPLC eluent was collected into mass spectrometer equipped, which recorded a precursor scan from m/z 500 to 1500 every 3.8 s. Phosphorylated peptides were sequenced by nanobore LC-ESMS/MS on a micromass quadrupole time-of-flight. Tandem MS data were collected every 2 s on a single precursor or alternating between a set of predefined precursors. The precursor selection window was set to 3 Da. Tryptic peptides for the phosphor site analysis were isolated by nano LC-MS and submitted for identification. The following day the samples were transferred with wild type and mutant IQGAP1 CTD constructs in Lipopectamine 2000 (Invitrogen) for 4 h as described previously (30). Cells were subsequently incubated at 37 °C, 5% CO2 in DMEM, 5% fetal calf serum for a further 16 h, fixed, and stained. Cells bearing neurites greater than 1 cell diameter were counted.
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**FIG. 1. IQGAP1 is a phosphoprotein.** IQGAP1 was isolated from MCF-7 cells grown in DMEM, 10% FBS and purified by SDS-PAGE. A Coomassie Blue-stained IQGAP1 band was excised from the gel and digested with trypsin. The tryptic digest was analyzed using a phosphopeptide-specific liquid chromatography-mass spectrometry technique in which the mass spectrometer detects phosphate released from phosphopeptides undergoing collision-induced dissociation in the ion source. The peak labeled IS is 500 fmol of a phosphopeptide internal standard spiked into the sample prior to analysis.

**RESULTS**

IQGAP1 Is a Phosphoprotein—Phosphorylation is a fundamental post-translational mechanism for regulating protein function. IQGAP1 contains numerous theoretical phosphorylation sites for several different protein kinases. To determine whether human IQGAP1 is phosphorylated in cells, we isolated IQGAP1 from MCF-7 human breast epithelial cells by calmodulin-Sepharose chromatography and SDS-PAGE. A Coomassie Blue-stained IQGAP1 band was excised from the gel and digested with trypsin. A small aliquot of the sample was analyzed by nanoelectrospray tandem mass spectrometry (25) to establish the protein as IQGAP1. The remaining sample was analyzed for phosphorylation content by using a phosphopeptide-specific liquid chromatography-mass spectrometry technique in which the mass spectrometer detects phosphate released from phosphopeptides undergoing collision-induced dissociation in the ion source (26, 32). These data demonstrated that endogenous IQGAP1 is phosphorylated on multiple sites in intact cells (Fig. 1).

**Phorbolester Promote IQGAP1 Phosphorylation in Intact Cells**—In silico analysis revealed that IQGAP1 has multiple consensus sequences for protein kinases, including CK2, protein kinase A, and PKC. Therefore, to determine whether basal phosphorylation could be augmented, MCF-7 cells were loaded with 32P and incubated with vehicle or phorbol ester, which activates PKC. When cells were serum-starved, minimal 32P incorporation was observed (Fig. 2A). In contrast, incubation for 20 min with PMA induced robust phosphorylation of endogenous IQGAP1 (Fig. 2A). Probing the blot with anti-IQGAP1 antibody verified that the phosphoprotein was IQGAP1 and that the amount of IQGAP1 was not increased in the PMA-treated sample.

To determine which region of IQGAP1 is phosphorylated, we separately transfected the N- and C-terminal halves of IQGAP1 into cells. Serum-starved cells were radiolabeled and incubated with PMA. Lysates were immunoprecipitated with anti-Myc antibody to isolate the transfected constructs. Autoradiography revealed that 32P was incorporated exclusively into the C-terminal half of IQGAP1; the N-terminal half was not phosphorylated (Fig. 2B). Western blotting revealed that the N- and C-terminal halves were present at equivalent levels. Identification of PMA-stimulated Sites of Phosphorylation on Endogenous IQGAP1—To identify specifically where endogenous IQGAP1 is phosphorylated in response to phorbol ester treatment, we performed mass spectrometry. Serum-starved MCF-7 cells were incubated with vehicle or PMA in the absence of 32P. Lysates were immunoprecipitated with anti-IQGAP1 antibody and purified by SDS-PAGE. After transfer to PVDF, membranes were processed by autoradiography (autorad) (to evaluate 32P incorporation) (left panel) followed by immunoblotting with anti-IQGAP1 antibody (right panel, WB). A, HER 293 cells were transiently transfected with Myc-tagged IQGAP1-N (N) or IQGAP1-C (C). Twentyeight h later, serum was removed for 18 h. Cells were loaded with 32P and incubated with PMA. Cell lysates were immunoprecipitated with anti-Myc antibody (the IQGAP1 constructs are Myc-tagged), and Western blots were processed by autoradiography (upper panel) followed by immunoblotting with anti-Myc antibody (lower panel). Data are representative of at least two independent experimental determinations.

**FIG. 2.** PMA stimulates IQGAP1 phosphorylation. A, MCF-7 cells were serum-starved, loaded with 32P, and incubated with PMA or vehicle (V) for 20 min. IQGAP1 was immunoprecipitated from cell lysates with anti-IQGAP1 antibody, and samples were resolved by SDS-PAGE. After transfer to PVDF, membranes were processed by autoradiography (autorad) (to evaluate 32P incorporation) (left panel) followed by immunoblotting with anti-IQGAP1 antibody (right panel, WB). B, HER 293 cells were transiently transfected with Myc-tagged IQGAPI-N (N) or IQGAPI-C (C). Twentyeight h later, serum was removed for 18 h. Cells were loaded with 32P, and incubated with PMA. Cell lysates were immunoprecipitated with anti-Myc antibody (the IQGAPI constructs are Myc-tagged), and Western blots were processed by autoradiography (upper panel) followed by immunoblotting with anti-Myc antibody (lower panel). Data are representative of at least two independent experimental determinations.
peaks yielded three phosphopeptide molecular weights 1785.4, 1913.4, and 700.2, which were tentatively assigned to IQGAP1 amino acid sequences 1441–1455, 1440–1455 or 1441–1456, and 1438–1442, respectively (Fig. 3D). LC-ESMS-based peptide sequencing of the triply charged ion (m/z 594.5) from the 1785-Da phosphopeptide (Fig. 3C and D) confirmed the sequence as 1441SKSVKEDSNLTLQEK1455. LC-ESMS-based sequencing of the 1913-Da phosphopeptide revealed two peptides with the sequences 1441–1456 and 1440–1455. The sequencing data for all three of these peptides conclusively identified Ser-1443 as the major site of phosphorylation. However, we were not able to rule out that a fraction of these sequences might be phosphorylated on Ser-1441. Supporting the notion that this serine was modified was the tentative assignment of the 700-Da phosphopeptide to the sequence MKKSK1442. This peptide would be produced efficiently only if Ser-1443 were not phosphorylated, thus allowing trypsin to cleave at Lys-1442 but not at Lys-1440 and Lys-1439. The relative abundance of the 700-Da phosphopeptide was rather low, suggesting either that this site is phosphorylated at low stoichiometry or that the peptide does not ionize well. We were not able to produce any direct sequencing data on this peptide. We could find no evidence for a peptide with phosphorylation at both Ser-1441 and Ser-1443.

To confirm that IQGAP1 is a direct substrate for PKC in cells, we preincubated serum-starved MCF-7 cells with or without the selective PKC inhibitor bisindolymaleimide (33) for 1 h and then stimulated the cells with phorbol esters. Phosphorylation-dependent LC-ESMS using full time precursor ion scanning on IQGAP1 from PMA-stimulated cells showed that the PKC inhibitor completely abolished phosphorylation on Ser-1443 (Fig. 4, bottom trace). In the absence of inhibitor, the IQGAP1 phosphorylation profile was as expected (Fig. 4, top trace).

**IQGAP1 Is a Substrate for PKC in Vitro**—PMA is widely used to activate PKC in cells. Therefore, we set out to determine whether IQGAP1 is a direct substrate for PKC. We first...
demonstrated that pure PKC catalyzed the phosphorylation of IQGAP1 in vitro. Incubation of IQGAP1 immunoprecipitated from serum-starved MCF-7 cells with PKC in the presence of \([\gamma-32P]ATP\) resulted in phosphorylation of IQGAP1 (Fig. 5A, lane 1). Although unlikely, it is possible that the radioactivity detected is a consequence of exchange of \(32P\) with phosphate on IQGAP1. In order to eliminate this possibility, we repeated the assay using GST-IQGAP1. The GST-IQGAP1 was expressed in \(E. coli\), thereby ensuring that the protein was not phosphorylated. As was observed with IQGAP1 isolated from cells, PKC catalyzed the phosphorylation of GST-IQGAP1 (Fig. 5A, lane 2). In addition, when phosphatidylserine (data not shown) or PKC (Fig. 5A, lanes 3 and 4) was omitted from the reaction mixture, no phosphate was incorporated into IQGAP1. Western blotting showed that the amounts of IQGAP1 protein in each sample did not differ substantially (Fig. 5A). Mass spectrometry identified Ser-1443 as the site phosphorylated by PKC. These data indicate that IQGAP1 is a substrate for PKC.

At least 11 isoenzymes of PKC have been identified in mammalian tissues (34). Inspection of the IQGAP1 amino acid sequence shows that Ser-1443 is contained within a PKC consensus motif (35). To determine whether IQGAP1 is a substrate for PKC, we incubated a GST fusion protein of wild type IQGAP1 in vitro with pure PKCs. Autoradiography revealed that PKC\(\alpha\) catalyzed the phosphorylation of IQGAP1 (Fig. 5B). PKC\(\alpha\) also catalyzed phosphorylation of the C-terminal half of IQGAP1 with a similar stoichiometry, whereas minimal phosphate was incorporated into the mutant IQGAP1 S1441A/S1443A (Fig. 5B). The Western blot demonstrates that loading of IQGAP1 protein was equivalent among samples. Analysis by mass spectrometry verified that PKC\(\alpha\) was able to catalyze phosphorylation of IQGAP1 in vitro on Ser-1443 (data not shown), the major site modified in cells after incubation with phorbol esters. We did not observe phosphorylation on Ser-1441 in vitro. These data confirm that PKC\(\alpha\) can be able to catalyze the phosphorylation of IQGAP1 in vitro, strongly supporting the notion that it is the kinase responsible for PMA-stimulated phosphorylation in intact cells.

**Distribution of IQGAP1 in Neuronal Cells**—In sessile cells IQGAP1 is distributed throughout the cytoplasm, with accumulation in the Golgi (36) and at cell-cell junctions (10). Moreover, IQGAP1 is expressed at the leading edge of motile cells (3, 8) and interacts with microtubules through CLIP-170 (12). The presence of IQGAP1 in neuronal cells has not been demonstrated previously. In order to document the expression of IQGAP1 in neuronal cells and to examine its subcellular distribution, hippocampal neurons from E17 rat embryos were immunostained with anti-IQGAP1 antibody (Fig. 6). This analysis revealed that IQGAP1 was present throughout the cell, along neurites and the developing axon as well as at the growth cone. Actin, which shows up the growth cones, exhibits some colocalization with IQGAP1. In addition, IQGAP1 showed colocalization with MAP-2 in immature neurites (Fig. 6). Because MAP-2 associates with microtubules, its colocalization with IQGAP1 suggests that IQGAP1 also associates with microtubules in hippocampal neurons.

**Effect of Phosphorylation on IQGAP1 Function**—In order to evaluate the potential functional sequelae of phosphorylation, we developed two mutant constructs of IQGAP1. Both Ser-1441 and Ser-1443 were converted by site-directed mutagenesis to Ala. This construct, termed IQGAP1 S1441A/S1443A, would not be phosphorylated in response to PMA. In the second construct, Ser-1441 and Ser-1443 were substituted by Glu and Asp, respectively. Termed IQGAP1 S1441E/S1443D, this construct has the serines replaced with negatively charged residues that may function in a phosphomimetic manner. Both mutant constructs were tagged at the N terminus with Myc, and transfected cells were identified with anti-Myc antibody. Wild type IQGAP1 was tagged at the N terminus with EGFP in

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**FIG. 5.** PKC catalyzes the phosphorylation of IQGAP1 in vitro. A. IQGAP1 immunoprecipitated from MCF-7 cells (lanes 1 and 3) or GST-IQGAP1 (expressed in \(E. coli\)) (lanes 2 and 4) was incubated with (+) or without (–) PKC in the presence of \([\gamma-32P]ATP\). Samples were processed by SDS-PAGE, Western blotting, and autoradiography (Autorad). Blots were probed with anti-IQGAP1 antibody. Note that the GST tag retards the migration of GST-IQGAP1. B. GST fusion constructs of wild type (WT) IQGAP1, the C-terminal (C) half of IQGAP1, and IQGAP1 S1441A/S1443A (AA) were incubated in vitro with (+) or without (–) PKC\(\alpha\) in the presence of \([\gamma-32P]ATP\). Samples were processed as described in A. All data are representative of at least two independent assays.
order to identify transfected cells. We verified by coimmunoprecipitation that the EGFP tag did not alter the interaction of IQGAP1 with several targets including calmodulin, Cdc42, β-catenin, and E-cadherin (data not shown).

The effects of phosphorylation on IQGAP1 function were examined in N1E-115 neuroblastoma cells. In culture these cells display a rounded morphology in the presence of serum (which activates Rho) but differentiate in response to Rac and Cdc42 activation (37). When plated on laminin, overexpression of Cdc42 effector proteins can overcome the serum block to induce neurites (38). For both nontransfected (data not shown) and mock-transfected cells, neurite outgrowth was observed in ~8% of the cells when grown in 5% fetal calf serum on laminin. Transfection of both wild type and mutant IQGAP1 constructs increased cell aggregation (data not shown). However, wild type IQGAP1 also slightly but significantly increased the proportion of cells with neurites (~14%) (Fig. 7). In contrast, cells that expressed IQGAP1 S1441A/S1443A failed to increase neurite outgrowth above control cells (Fig. 7). The most dramatic effects were observed with the phosphomimetic IQGAP1 construct. On transfection with IQGAP1 S1441E/S1443D, ~30% of N1E-115 cells showed neurite outgrowth (Fig. 7). These data suggest that PMA-stimulated phosphorylation of IQGAP1 significantly enhances its ability to induce neurite outgrowth in N1E-115 cells.

**DISCUSSION**

IQGAP1 is becoming recognized as an important regulator of the cytoskeleton (2). The effects of IQGAP1 on the actin cytoskeleton are mediated both by a direct interaction with actin and indirectly via Cdc42 and Rac1. IQGAP1 promotes F-actin polymerization in vitro and colocalized with actin in membrane ruffles (8, 15). Although the name implies that it functions as a GTPase-activating protein, IQGAP1 actually stabilizes Cdc42 and Rac1 in the active, GTP-bound form (5, 8, 17). Thus, IQGAP1 acts as an “anti-GTPase-activating protein” for Cdc42 and Rac1, with marked effects on the cytoskeleton. For example, overexpressing IQGAP1 increased the amount of active Cdc42 in cells, promoting the formation of filopodia (17). Conversely, a dominant negative IQGAP1 prevented bradykinin from activating Cdc42 and from inducing filopodia formation (17). Moreover, IQGAP1 promoted cell migration and invasion in a Cdc42- and Rac1-dependent manner (3). IQGAP1 also modulates microtubule function via binding to the microtubule tip protein CLIP-170 (12). In this study we document that phosphorylation of IQGAP1 provides an additional mechanism by which it can regulate cytoskeletal function.

IQGAP1 is highly phosphorylated in MCF-7 cells. When isolated from cells starved of serum, the extent of phosphorylation is reduced (see Fig. 3A). Therefore, it is reasonable to infer that growth factors (and perhaps other serum components) promote IQGAP1 phosphorylation. In this work we demonstrate that PMA induces phosphate incorporation into Ser-1441 and Ser-1443 of IQGAP1. Several factors point to PKC as the kinase responsible for catalyzing this phosphorylation. First, PMA is widely recognized as an activator of endogenous PKC (39) and has been used extensively to examine the biological roles of PKC. Second, Ser-1441 and especially Ser-1443 reside in characteristic PKC consensus sequences (35). Although Ser-1443 is a hypothetical CK2 phosphorylation site (consensus (S/T)XXD/E) (40), the presence of the basic Lys at residue 1445 renders IQGAP1 Induces Neurite Outgrowth.

**FIG. 6.** IQGAP1 is expressed in differentiating hippocampal neurons. Hippocampal neurons from E17 rat embryos cultured for 48 h were stained with anti-IQGAP1 antibody and Cy5-conjugated secondary antibody, with anti-MAP-2 antibody (Sigma) and fluorescein-conju-
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IQGAP1 is a multidomain protein containing a number of protein-interacting motifs through which it binds target molecules (1). The two residues phosphorylated by PKC, Ser-1441 and Ser-1443, are located between the RasGAP-related domain, required for Cdc42 and Rac1 binding, and the RasGAP_C. No proteins are known to bind directly to this region. Nevertheless, there are a number of possible mechanisms by which phosphorylation at Ser-1441 and Ser-1443 may alter the function of IQGAP1. These mechanisms are not mutually exclusive, and more than one may contribute to our observations. Phosphate incorporation may directly modify the interaction of an unidentified IQGAP1 binding partner. Alternatively, phosphorylation may alter the tertiary conformation of IQGAP1, thereby changing its interaction with cytoskeleton. A third possibility is that phosphorylation may regulate IQGAP1 function by affecting dimerization. The last two postulated mechanisms have been documented for ezrin/radixin/moesin. Phosphorylation of ezrin/radixin/moesin by PKC changes its conformation, facilitating binding to actin, and also disrupts oligomerization (42).

The presence of IQGAP1 in neurons and its colocalization with MAP-2 is documented here. Most interestingly, overexpression of IQGAP1 promotes neurite outgrowth. In hippocampal neurons atypical PKCs (PKCζ and PKCδ) in association with Par6 and Cdc42/Rac play an important role in establishing neuronal polarity (43, 44). PKCζ specifically regulates receptor trafficking and extracellular signal-regulated kinase signaling in hepatocyte growth factor/c-Met-induced cell migration (45). In neuroblastoma cells, PKCζ can promote neurite outgrowth through interactions of its regulatory domain (46). Rac activation may also be mediated by Ca^{2+} and by phosphorylation of RhoGDI by conventional PKC (47). These effects of PKCs make it difficult to dissect out the role of IQGAP1. Therefore, the strategy we adopted to identify the specific effects mediated by PKC-induced phosphorylation of IQGAP1 was to develop and use phosphorylation-site mutants of IQGAP1. This approach revealed very significant effects of phosphorylation on IQGAP1 function. In fact, the magnitude of the effect on neurite outgrowth produced by the phosphomimetic IQGAP1 S1441D/S1443E was similar to that generated by expression of Cdc42-associated proteins such as IRSp53 (38). The molecular mechanism by which IQGAP1 promotes neurite outgrowth, and the enhanced effect of IQGAP1 S1441E/S1443D, is not known. In view of the diverse mechanisms by which IQGAP1 modulates the cytoskeleton, there are several targets, including actin, Cdc42/Rac1, and microtubules that are potential candidates for mediating its neuronal effects. Based on our prior observations that IQGAP1 promotes cell migration in a Cdc42- and Rac1-dependent manner (3), it is tempting to speculate that these GTPases mediate the neurite outgrowth demonstrated here. Further work is needed to address this hypothesis.

In summary, the data presented here reveal for the first time that IQGAP1 is highly phosphorylated in human cells. Moreover, IQGAP1 is a substrate for PKC both in vitro and in intact cells. We also disclose a previously unidentified role for IQGAP1 in neurite outgrowth. Finally, phosphorylation by PKC significantly augments the ability of IQGAP1 to promote neurite outgrowth, providing an additional level of complexity to the multiple mechanisms by which IQGAP1 regulates the cytoskeleton.

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Note Added in Proof—During the review process Ser-1443 was confirmed as a PKC-catalyzed phosphorylation site on IQGAP1 (49).

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