

# Glycogen Synthase Kinase-3 Phosphorylates CdGAP at a Consensus ERK 1 Regulatory Site\*

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Rho GTPases regulate a multitude of cellular processes from cytoskeletal reorganization to gene transcription and are negatively regulated by GTPase-activating proteins (GAPs). Cdc42 GTPase-activating protein (CdGAP) is a ubiquitously expressed GAP for Rac1 and Cdc42. In this study, we set out to identify CdGAP-binding partners and, using a yeast two-hybrid approach, glycogen synthase kinase 3 $\alpha$  (GSK-3 $\alpha$ ) was identified as a partner for CdGAP. GSK-3 exists in two isoforms,  $\alpha$  and  $\beta$ , and is involved in regulating many cellular functions from insulin response to tumorigenesis. We show that GSK-3 $\alpha$  and - $\beta$  interact with CdGAP in mammalian cells. We also demonstrate that GSK-3 phosphorylates CdGAP both *in vitro* and *in vivo* on Thr-776, which we have previously shown to be an ERK 1/2 phosphorylation site involved in CdGAP regulation. We report that the mRNA and protein levels of CdGAP are increased upon serum stimulation and that GSK-3 activity is necessary for the up-regulation of the protein levels of CdGAP but not for the increase in mRNA. We conclude that GSK-3 is an important regulator of CdGAP and that regulation of CdGAP protein levels by serum presents a novel mechanism for cells to control Cdc42/Rac1 GTPase signaling pathways.

The Rho subfamily of small GTPases controls a wide variety of cellular functions. RhoA, Rac1, and Cdc42 are the best known members of this family, and they are most often associated with their roles as regulators of cytoskeleton remodeling and as key mediators of the activation of transcription of genes downstream of growth factor receptors (1). Much evidence exists linking Rho GTPases to transformation of cells; however, contrary to the Ras gene, activating mutations in Rho genes are rarely found in human cancers (2, 3). It seems instead that the expression of Rho GTPases and expression and function of regulators of the Rho subfamily of GTPases are altered during cellular transformation (2, 3).

Rho GTPases act in a cycle as molecular switches with an active GTP-bound form and an inactive GDP-bound form (1). The GTPase-activating proteins (GAPs)<sup>5</sup> negatively regulate the GTPases by enhancing the hydrolysis of GTP to GDP (1). To date, ~70 human genes are predicted to encode for potential RhoGAP proteins (4), which is roughly triple the number of Rho GTPases (1). This lends weight to the notion that regulators like the RhoGAPs tightly control Rho GTPases and lend context-dependent specificity to their processes. Thus, the activity of RhoGAPs must be highly regulated in both spatial and temporal fashions. RhoGAPs are regulated at the protein level by a variety of mechanisms ranging from protein-protein interactions to phosphorylation, lipid interactions, and proteolytic degradation (5).

Cdc42 GTPase-activating protein (CdGAP) has been shown to regulate both Cdc42 and Rac1 *in vitro* and *in vivo* and exists in two main isoforms: a short form of 820 amino acids containing an N-terminal RhoGAP domain, a central region, and a C-terminal proline-rich region (PRD) (6, 7) and a long isoform comprising the entire short form with an additional C-terminal region extending to 1425 amino acids total (8). Recently, we demonstrated that CdGAP activity is negatively controlled by protein-protein interactions via the endocytic protein intersectin (6) and by phosphorylation within its PRD (8).

In this study, we used a yeast two-hybrid approach to look for binding partners for the PRD of CdGAP and found glycogen synthase kinase-3 $\alpha$  (GSK-3 $\alpha$ ) as an interacting partner. GSK-3 $\alpha$  and its closely related isoform GSK-3 $\beta$  are serine/threonine protein kinases initially identified as key enzymes in the regulation of glycogen metabolism by insulin and are now known to be implicated in many diverse cellular processes including tumorigenesis, cell survival, and developmental patterning (9, 10). We demonstrate that GSK-3 $\beta$  can also bind CdGAP. We further report that GSK-3 phosphorylates CdGAP *in vivo* under serum-starved conditions where GSK-3 is most active. GSK-3 phosphorylates CdGAP both *in vitro* and *in vivo* at Thr-776, which we have previously shown to be an ERK1/2 phosphorylation site involved in CdGAP regulation (8). We demonstrate that the mRNA and protein levels of CdGAP are up-regulated in response to serum in a transcriptionally mediated manner and that GSK-3 activity is critical in the up-regulation of protein levels but not mRNA levels.

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<sup>5</sup> The abbreviations used are: GAP, GTPase-activating protein; CdGAP, Cdc42 GTPase-activating protein; CdGAP-l, CdGAP long isoform; CdGAP-s, CdGAP short isoform; PRD, proline-rich domain; GSK-3, glycogen synthase kinase 3; ActD, actinomycin D; DRB, 5,6-dichlorobenzimidazole 1- $\beta$ -D-ribofuranoside; MBP, myelin basic protein; MOPS, 4-morpholinepropanesulfonic acid.

## EXPERIMENTAL PROCEDURES

**Reagents and Antibodies**—Lithium chloride, AR-A014418, SB 415286, actinomycin D (ActD), and 5,6-dichlorobenzimidazole 1- $\beta$ -D-ribofuranoside (DRB) were purchased from Sigma Canada, and sodium chloride was from Fisher. Anti-GSK-3 $\alpha$  antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-GSK-3 $\beta$  antibody was from Cell Signaling Technologies, anti-GSK3 (both  $\alpha$  and  $\beta$ ) antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-phospho-GSK-3 antibody, anti-phospho-Ser-641 glycogen synthase, and anti-glycogen synthase antibodies were from Cell Signaling Technologies. [ $^{32}$ P]ATP (3,000 Ci/mmol) and [ $^{32}$ P]orthophosphate (3,000  $\mu$ Ci/ml) were purchased from PerkinElmer Life Sciences. Recombinant GSK-3 $\alpha$  and - $\beta$  and phospho-glycogen synthase peptide were purchased from Upstate Biotechnology. Polyclonal anti-CdGAP antibodies were produced and purified as described previously (8, 11). Polyclonal antibodies against phospho-Thr-776 of CdGAP were produced by immunizing rabbits with a peptide CXPPPPT\*PLEEEPE (T\* represents phosphorylated threonine, and X represents hexanoic acid spacer). Serum from the immunized rabbits was taken and affinity-purified against the same peptide. This fraction was taken and run through a column containing a non-phosphorylated peptide CXPPPPTPLEEEPE, and the flow-through (antibody unable to bind the non-phosphorylated residue) was kept. Myc-tagged proteins were detected by using the 9E10 anti-Myc monoclonal antibody, which was kindly provided by Dr. Nicole Beauchemin (McGill University, Montreal, Canada).

**DNA Constructs**—CdGAP constructs were as described previously (6, 8, 11). GSK-3 $\alpha$  was obtained as a full clone in the pACTII vector (see “Yeast Two-hybrid Screen”). It was subcloned into pRK5 vector using the XhoI/EcoRI restriction sites. GSK-3 $\beta$  cDNA was kindly provided by Dr. James Woodgett (Ontario Cancer Institute, Toronto, Canada).

**Yeast Two-hybrid Screen**—The PRD of CdGAP (amino acids 516–820) was fused to the GAL4 DNA-binding domain (using a pYTH6 vector) and stably integrated into the genome of the Y190 strain of *Saccharomyces cerevisiae*. This was used to screen a library of human brain cDNAs fused to the GAL4 activation domain (pACTII vector) (a kind gift from Dr. Alan Hall, Sloan-Kettering, New York, NY) (12), as described previously (13). Approximately  $6 \times 10^6$  clones were screened for their ability to grow on selective medium containing 25 mM 3-amino-1,2,4-triazole. The 80 fastest growing clones were replated, and plasmids were isolated using the Wizard clean-up kit (Promega) and retransformed into the CdGAP-PRD yeast strain. The clones growing on selective medium and positive for  $\beta$ -galactosidase activity were sequenced. Six clones corresponded to the entire coding sequence of GSK-3 $\alpha$ .

**Cell Transfection and Immunoprecipitation**—HEK-293, NIH 3T3, and U2OS cells (the latter kindly provided by Dr. Christopher E. Turner, SUNY Upstate Medical University, Syracuse, NY) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics and maintained at an atmosphere of 5% CO<sub>2</sub> at 37 °C. HEK-293 cells were transfected using Lipofectamine (Invitrogen) according to

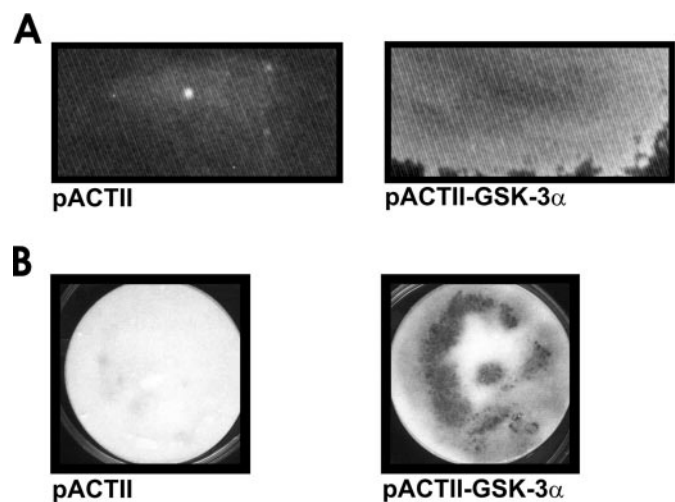
the manufacturer's protocol. Briefly, 2  $\mu$ g total of DNA (1  $\mu$ g of either GSK-3 $\alpha$  or GSK-3 $\beta$  along with 1  $\mu$ g of empty vector, pRK5myc-CdGAP-s, pRK5myc-CdGAP-l, or pRK5myc-CdGAP-PRD) was transfected per 60-mm dish. Cells were lysed 24 h after transfection in 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g of aprotinin/ml, 10  $\mu$ g of leupeptin/ml, 20 mM NaF, and 1 mM sodium orthovanadate followed by centrifugation for 15 min at  $1,000 \times g$ . Then, 1 mg of the resulting postnuclear supernatant was incubated overnight at 4 °C with 5  $\mu$ g of anti-Myc antibodies and 25  $\mu$ l of 50% protein G-Sepharose (Amersham Biosciences). Samples were washed three times in lysis buffer and subjected to SDS-PAGE followed by immunoblotting analysis with anti-Myc, anti-GSK-3 $\alpha$ , or anti-GSK-3 $\beta$  antibodies. NIH 3T3 cells were plated at a density of  $4 \times 10^5$  cells/100-mm dish. Twenty-four hours later, cells were serum-starved for 20 h followed by stimulation with 15% serum for various periods of time. The cells were lysed as described above, and the amount of protein was quantified using a Pierce BCA kit. Equal amounts of total protein from the various stimulation conditions were submitted to SDS-PAGE and Western blotting. U2OS cells were plated at a density of  $2 \times 10^6$  cells/100-mm dish. Twenty-four hours later, cells were serum-starved for 20 h followed by a 4-h stimulation with GSK-3 inhibitors. The cells were lysed as described above, and the amount of protein was quantified using a Pierce BCA kit. Equal amounts of total protein were submitted to SDS-PAGE and Western blotting.

**In Vivo [ $^{32}$ P]orthophosphate Labeling**—NIH 3T3 cells were serum-starved overnight. The cells were either left untreated or were treated with 50 mM LiCl, 50 mM NaCl, or 0.1% Me<sub>2</sub>SO or with 100  $\mu$ M SB 415286 or AR-A014418 for 1 h. This was followed by a 1-h incubation in either phosphate-free medium or phosphate-free medium treated with the above-mentioned compounds for 1 h. Cells were then incubated for 2 h in phosphate-free medium supplemented with 0.5 mCi of [ $^{32}$ P]orthophosphate/ml, with or without treatment with the above mentioned compounds. The cells were lysed, and CdGAP was immunoprecipitated using polyclonal anti-CdGAP antibodies (8) overnight at 4 °C. The samples were submitted to SDS-PAGE followed by transfer to nitrocellulose and then autoradiography and Western blotting against CdGAP.

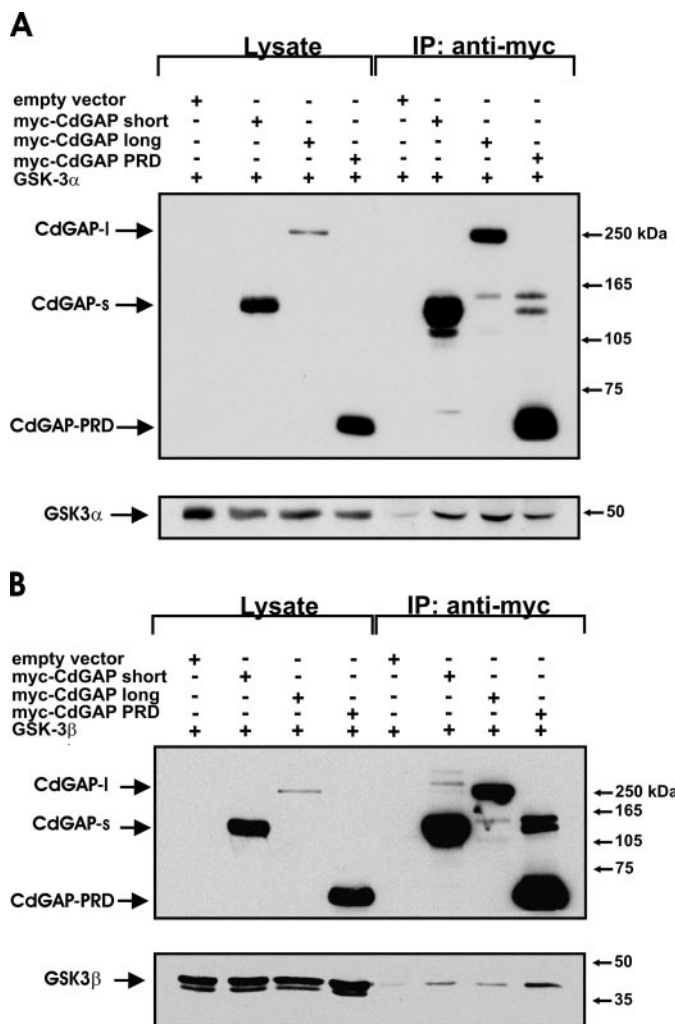
**In Vitro Kinase Assay and Phospho-Amino Acid Analysis**—Hexahistidine fusion proteins—CdGAP-PRD or CdGAP-PRD-T776A were produced and purified as described previously (8). His-tagged CdGAP-PRD or CdGAP-PRD-T776A were incubated with 10 ng of active GSK-3 $\alpha$  or GSK-3 $\beta$  (Upstate Biotechnology) in 8 mM MOPS, pH 7.0, 200 nM EDTA, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M ATP, and 10  $\mu$ Ci/ml [ $\gamma$ - $^{32}$ P]ATP for 10 min at 30 °C. The reaction was stopped by the addition of Laemmli buffer. The samples were submitted to SDS-PAGE and transferred to nitrocellulose or polyvinylidene difluoride membrane, and phosphorylated proteins were visualized by autoradiography. A range of CdGAP-PRD concentrations from 18.2 nM to 9.32  $\mu$ M were used to estimate the  $K_m$  value using the Lineweaver-Burk equation (14). Phospho-amino acid analysis was performed as described previously (8, 15).

**Quantitative Reverse Transcription-PCR**—NIH 3T3 cells were serum-starved overnight. The cells were either left

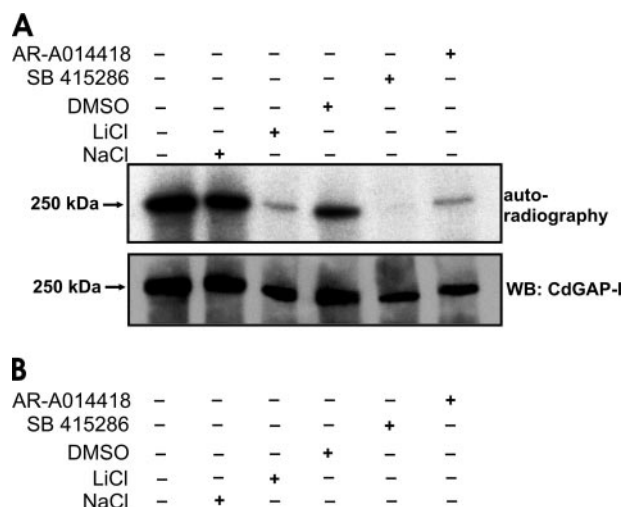
## GSK-3 Regulates CdGAP



**FIGURE 1. CdGAP interacts with GSK-3 in a yeast two-hybrid screen.** A Y190 yeast strain stably expressing Gal4 DNA-binding domain fused to CdGAP-PRD was transformed with pACTII or pACTII-GSK-3 $\alpha$ . *A*, growth on 3-amino-1,2,4-triazole medium plates. *B*,  $\beta$ -galactosidase assay (shown after 3 h of incubation).



**FIGURE 2. CdGAP interacts with GSK-3 in mammalian cells.** GSK-3 $\alpha$  (*A*) and GSK-3 $\beta$  (*B*) were co-transfected with either pRK5myc vector or pRK5myc vector containing CdGAP-s, CdGAP-l, or CdGAP-PRD into HEK 293 cells. Anti-Myc immunoprecipitations (*IP*) were carried out followed by SDS-PAGE and transfer to nitrocellulose membrane. Western blots were performed against the Myc epitope tag and GSK-3 $\alpha$  (*A*) or GSK-3 $\beta$  (*B*).



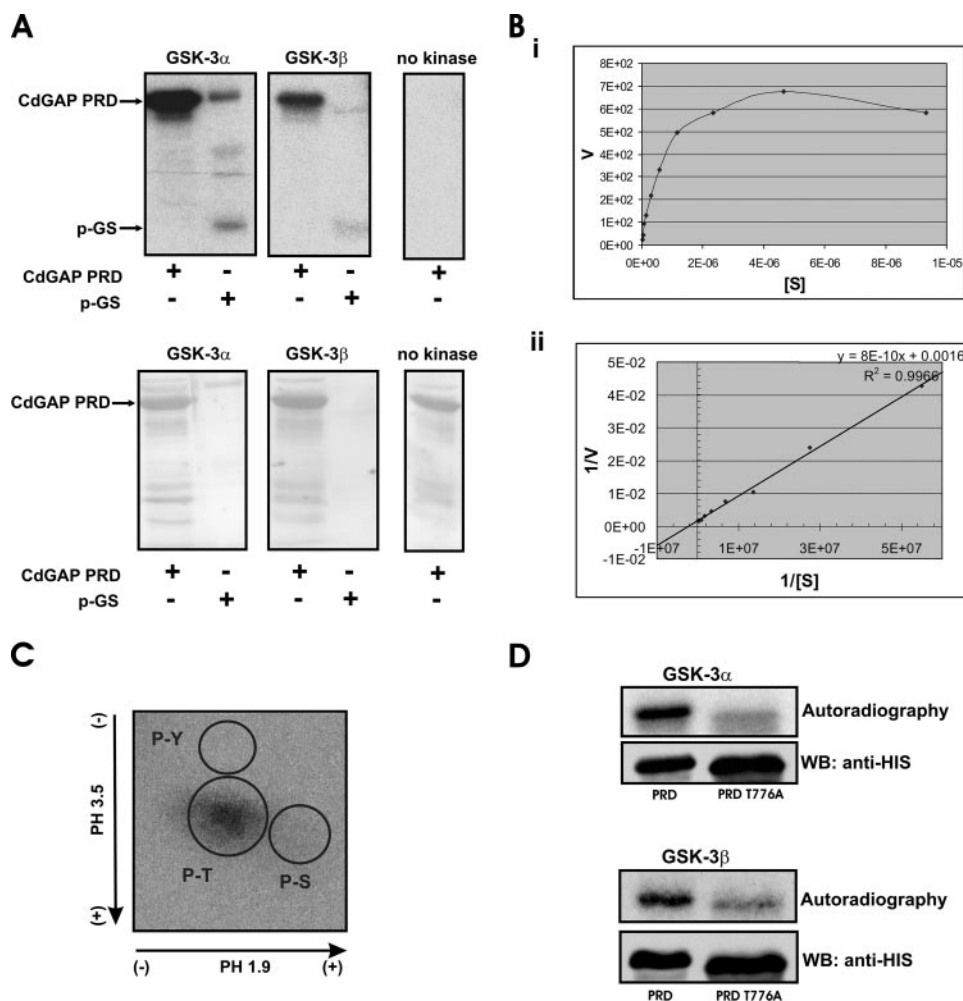
**FIGURE 3. CdGAP is phosphorylated *in vivo* by GSK-3.** *A*, NIH 3T3 cells were serum-starved overnight and then incubated in serum free medium with or without 50 mM NaCl, 50 mM LiCl, 0.1% Me<sub>2</sub>SO (*DMSO*), 100  $\mu$ M SB 415286, or 100  $\mu$ M AR-A014418 prior to and during metabolic labeling with 0.5 mCi of [<sup>32</sup>P]orthophosphate/ml. CdGAP was immunoprecipitated and submitted to SDS-PAGE followed by transfer to nitrocellulose and autoradiography (*top panel*) and Western blotting (*WB*) using anti-CdGAP antibodies (*bottom panel*). *B*, NIH 3T3 cells were treated under similar conditions as above, and protein lysates were submitted to SDS-PAGE followed by Western blotting against phospho-Ser-641 glycogen synthase (*GS*), glycogen synthase, phospho-Ser-9 GSK-3 $\beta$ , GSK-3 $\alpha$  and - $\beta$ , and  $\beta$ -actin.

untreated or treated with 15% serum or with 15% serum with transcriptional or GSK-3 inhibitors for various times. Total RNA was extracted using a Qiagen RNeasy kit (Qiagen). mRNA was reverse-transcribed using enzymes from Invitrogen. The cDNA was then run in a quantitative real-time PCR reaction using a Roche Applied Science Lightcycler, Qiagen Quantitect Sybr green reagents, and CdGAP primers obtained from Gene-globe. 18 S ribosomal subunit primers (a kind gift from Dr. Simon Wing, McGill University) were used as a loading control.

## RESULTS

The PRD of CdGAP harbors five consensus SH3-binding motifs and is known to be necessary in the regulation of CdGAP activity (6). To identify target proteins that interact with CdGAP through this region of the protein, we used the PRD (amino acids 516–820) as bait in a yeast two-hybrid screen with a human brain cDNA library. GSK-3 $\alpha$  was isolated as a positive clone growing on selective medium (Fig. 1*A*) and expressing  $\beta$ -galactosidase (Fig. 1*B*). To examine whether CdGAP interacts with both GSK-3 $\alpha$  and GSK-3 $\beta$  in mammalian cells, Myc-tagged CdGAP-long (CdGAP-l), short (CdGAP-s), or PRD





**FIGURE 4. CdGAP is phosphorylated *in vitro* on Thr-776 by GSK-3.** *A*, an *in vitro* kinase assay was performed using active GSK-3 $\alpha$  (left panel), GSK-3 $\beta$  (middle panel), or no kinase (right panel) and either His-CdGAP-PRD or phospho-glycogen synthase (p-GS) followed by SDS-PAGE, transfer to nitrocellulose, and autoradiography (top panels) or Ponceau-S staining (bottom panels). *B*, an *in vitro* kinase assay was performed using recombinant GSK-3 $\alpha$  and concentrations of His-CdGAP-PRD ranging from 18.2 nM to 9.32  $\mu$ M. Incorporation of [ $^{32}$ P]phosphate into CdGAP-PRD was determined by measuring the CPM. A Michaelis-Menten plot (i) was constructed by plotting  $V$  ( $V$  = CPM/minute) against  $[S]$  ( $[S]$  = concentration of CdGAP-PRD in moles/liter), and a Lineweaver-Burk plot (ii) was constructed by plotting  $1/V$  against  $1/[S]$ . *C*, *in vitro* phosphorylated His-CdGAP-PRD was hydrolyzed and submitted to phospho-amino acid analysis. Migration of phospho-amino acid standards is indicated with circles; substances used are as follows: phospho-serine (P-S), phospho-threonine (P-T), and phosphotyrosine (P-Y). *D*, *in vitro* kinase assay using either GSK-3 $\alpha$  or  $\beta$  with His-CdGAP-PRD and His-CdGAP-PRD T776A. Samples were resolved by SDS-PAGE and were transferred to nitrocellulose followed by autoradiography and Western blotting (WB) using anti-His antibodies.

(CdGAP-PRD) and GSK-3 $\alpha$  (Fig. 2A) or GSK-3 $\beta$  (Fig. 2B) were transfected into HEK-293 cells, and CdGAP-s, CdGAP-l, and CdGAP-PRD were immunoprecipitated using anti-Myc antibodies. As shown in Fig. 2, A and B, both GSK-3 $\alpha$  and GSK-3 $\beta$  co-immunoprecipitated with CdGAP-s and -l, as well as with the PRD of CdGAP in mammalian cells. Taken together, these data indicate that CdGAP interacts with both GSK-3 $\alpha$  and GSK-3 $\beta$ .

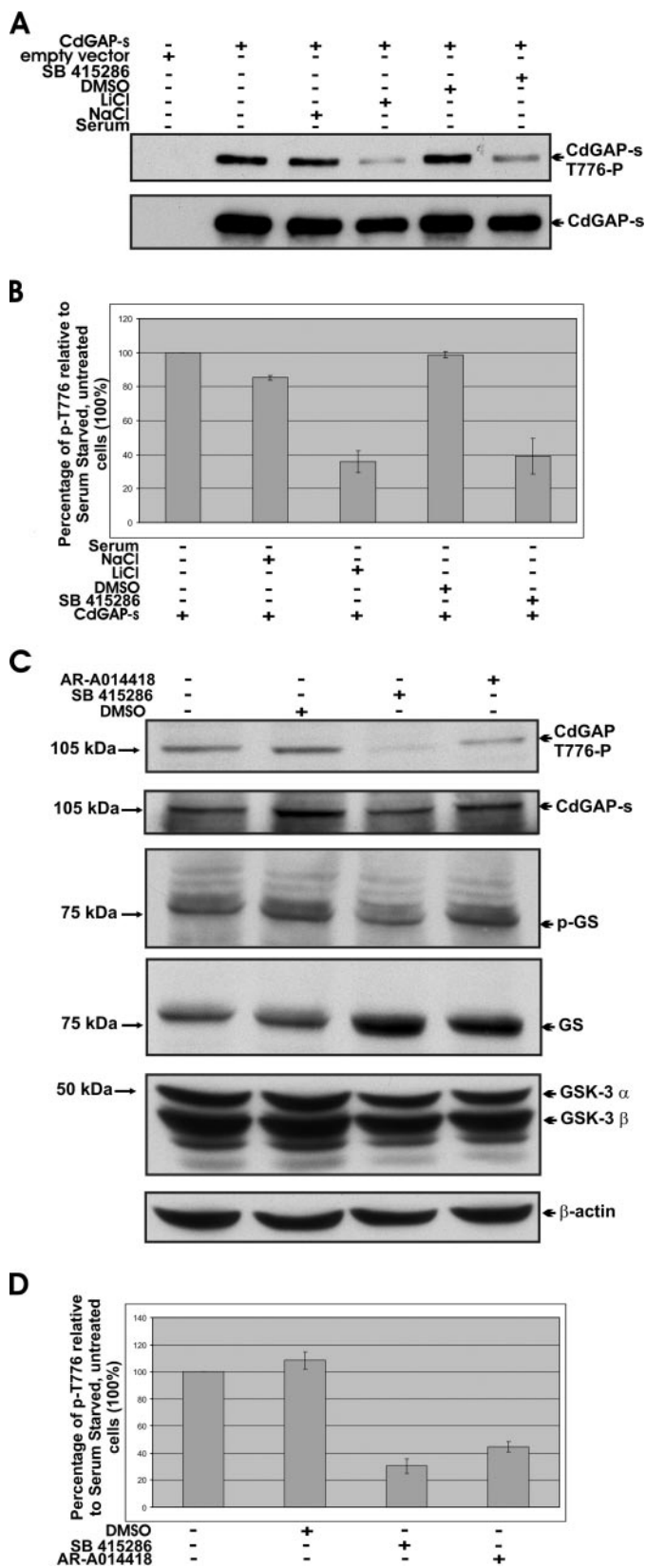
We have previously shown that both CdGAP-s and CdGAP-l are highly phosphorylated on serine and threonine residues in fibroblasts (8, 11). Moreover, we have demonstrated that CdGAP-l is *in vivo* phosphorylated at a basal level in serum-starved conditions where GSK-3 is mostly active (8, 10), and further phosphorylation is stimulated via the mitogen-activated protein kinase (MAPK) pathway in response to serum (8).

To investigate whether endogenous CdGAP phosphorylation is mediated by GSK-3, NIH 3T3 cells endogenously expressing the long isoform of CdGAP (CdGAP-l) were serum-starved overnight and were then left untreated or were treated with the GSK-3 inhibitors LiCl (9), SB 415286 (16), or AR-A014418 (17) for 1 h prior to incubation in phosphate-free medium supplemented with [ $^{32}$ P]orthophosphate. As expected, GSK-3 activity was inhibited as indicated by Western blotting for phospho-Ser-641 of glycogen synthase, a known GSK-3 substrate (18) (Fig. 3B). Additionally, inhibition of GSK-3 by LiCl is indicated by the increase in phospho-serine 9 of GSK-3, a site whose phosphorylation is affected by LiCl but not the SB 415286 or AR-A014418 inhibitors (19). CdGAP-l was phosphorylated under serum-starved conditions where GSK-3 was active, as well as when sodium chloride and Me<sub>2</sub>SO were present as negative controls; however, its phosphorylation was significantly reduced by the three GSK-3 inhibitors (Fig. 3A). Therefore, these results show that CdGAP-l is *in vivo* phosphorylated by GSK-3 in NIH 3T3 cells.

We next tested whether GSK-3 phosphorylates CdGAP *in vitro*. Since we know that most of the phosphorylation of CdGAP is in the PRD (8), we performed *in vitro* kinase assays with recombinant His-tagged CdGAP-PRD incubated with activated GSK-3 $\alpha$  or  $\beta$ . As shown in Fig. 4A, both GSK-3 $\alpha$  and GSK-3 $\beta$  were able to phosphorylate

CdGAP-PRD *in vitro*. Using a range of CdGAP-PRD concentrations from 18.2 nM to 9.32  $\mu$ M, we estimated a  $K_m$  value of 0.5  $\mu$ M, showing that CdGAP-PRD is a very good substrate for GSK-3 (Fig. 4B). To determine which types of residues are phosphorylated by GSK-3, CdGAP-PRD phosphorylated *in vitro* by GSK-3 $\alpha$  was used to perform a phospho-amino acid analysis. We found that CdGAP-PRD is mainly phosphorylated on threonine residues (Fig. 4C). The predicted consensus phosphorylation motif for GSK-3 consists of (S/T)XXX(pS/pT) (20), in which a proline is a preferred residue adjacent to the phosphorylation site, and in most cases, a "primed" phosphorylation site is required at the +4 position prior to GSK-3 phosphorylation of its substrates (20). However, several targets of GSK-3 bypass the need for the +4 phosphorylation by substituting a charged residue at this site (21, 22). This is of particular interest

## GSK-3 Regulates CdGAP



**FIGURE 5. CdGAP is phosphorylated *in vivo* on Thr-776 by GSK-3.** *A*, NIH 3T3 cells transfected with empty vector or CdGAP-s were serum-starved overnight and were either left untreated or treated with 50 mM NaCl, 50 mM LiCl, 0.1% Me<sub>2</sub>SO (DMSO), or 100 μM SB 415286 for 5 h. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP phospho-Thr-776 (top panel). The membrane was stripped and reprobed with anti-CdGAP antibodies (bottom panel). *B*, quantitative analysis of *A* representing

since GSK-3 efficiently phosphorylates CdGAP-PRD *in vitro*, whereas targets of GSK-3 that need priming generally make poor *in vitro* substrates of GSK-3, indicating that it may not need to be primed. CdGAP contains one atypical motif, within the proline-rich domain, <sup>776</sup>TPLEE<sup>780</sup>. We mutated Thr-776 to alanine and determined by *in vitro* kinase assays that its phosphorylation by GSK-3α and -β was significantly reduced when compared with wild-type CdGAP-PRD (Fig. 4D). Interestingly, we have previously demonstrated that Thr-776 is a target site of ERK1/2 and acts as an important regulatory site of CdGAP activity *in vivo* (8).

We then developed a polyclonal antibody against CdGAP phospho-Thr-776. This antibody was able by Western blotting to recognize Myc-tagged CdGAP-s protein overexpressed in COS-7 fibroblasts but was unable to recognize both Myc-tagged CdGAP-s T776A and recombinant CdGAP-PRD expressed in *Escherichia coli* (data not shown). When Myc-tagged CdGAP-s was expressed in serum-starved NIH 3T3 cells, we found that CdGAP-s was phosphorylated on Thr-776 (Fig. 5, *A* and *B*). However, when these cells were incubated with LiCl for 5 h, the level of CdGAP-s with phosphorylated Thr-776 was markedly decreased relative to both serum-starved conditions, as well as cells that were incubated with NaCl for 5 h (Fig. 5, *A* and *B*). Likewise, when cells were incubated with the GSK-3 inhibitor SB 415286 (16) for 5 h, the level of phospho-Thr-776 CdGAP-s was markedly decreased relative to both serum-starved conditions and cells that were incubated instead with Me<sub>2</sub>SO (Fig. 5, *A* and *B*). To examine whether Thr-776 of endogenous CdGAP is phosphorylated *in vivo* by GSK-3, U2OS osteosarcoma cells endogenously expressing the short isoform of CdGAP (23) were serum-starved overnight and then left untreated or were treated with the GSK-3 inhibitors SB 415286 or AR-A014418. We found that CdGAP-s was phosphorylated on Thr-776 under serum-starved conditions; however, CdGAP-s from cells that were incubated with the GSK-3 inhibitors showed a dramatic decrease in phosphorylation at this site (Fig. 5, *C* and *D*). As expected, the decrease in the levels of phospho-Ser-641 glycogen synthase relative to total glycogen synthase confirmed that GSK-3 activity was inhibited (Fig. 5C). Altogether, these results indicate that GSK-3 phosphorylates *in vivo* the residue Thr-776 of CdGAP-s.

GSK-3 regulates a great deal of cellular functions, including gene expression and protein stability (10). To determine whether GSK-3 affects CdGAP protein levels, we first examined the levels of endogenous CdGAP-I proteins in subconfluent NIH 3T3 fibroblasts stimulated with serum for various periods of time. Interestingly, we observed that CdGAP-I protein levels were significantly augmented in response to serum (Fig. 6, *A* and *B*). This induction occurred as early as 1 h after stim-

CdGAP phospho-Thr-776 relative to serum-starved, untreated cells. *C*, U2OS cells were serum-starved overnight and were either left untreated or treated with 0.1% Me<sub>2</sub>SO, 100 μM SB 415286, or 100 μM AR-A014418 for 4 h. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP phospho-Thr-776 (top panel). The membrane was stripped and reprobed with anti-CdGAP antibodies (second panel). Lysates were also probed for levels of phospho-Ser-641 glycogen synthase (GS), glycogen synthase, GSK-3, and β-actin. *D*, quantitative analysis of *C* representing CdGAP phospho-Thr-776 relative to serum-starved, untreated cells. Error bars represent standard errors of the mean relative to three independent experiments.

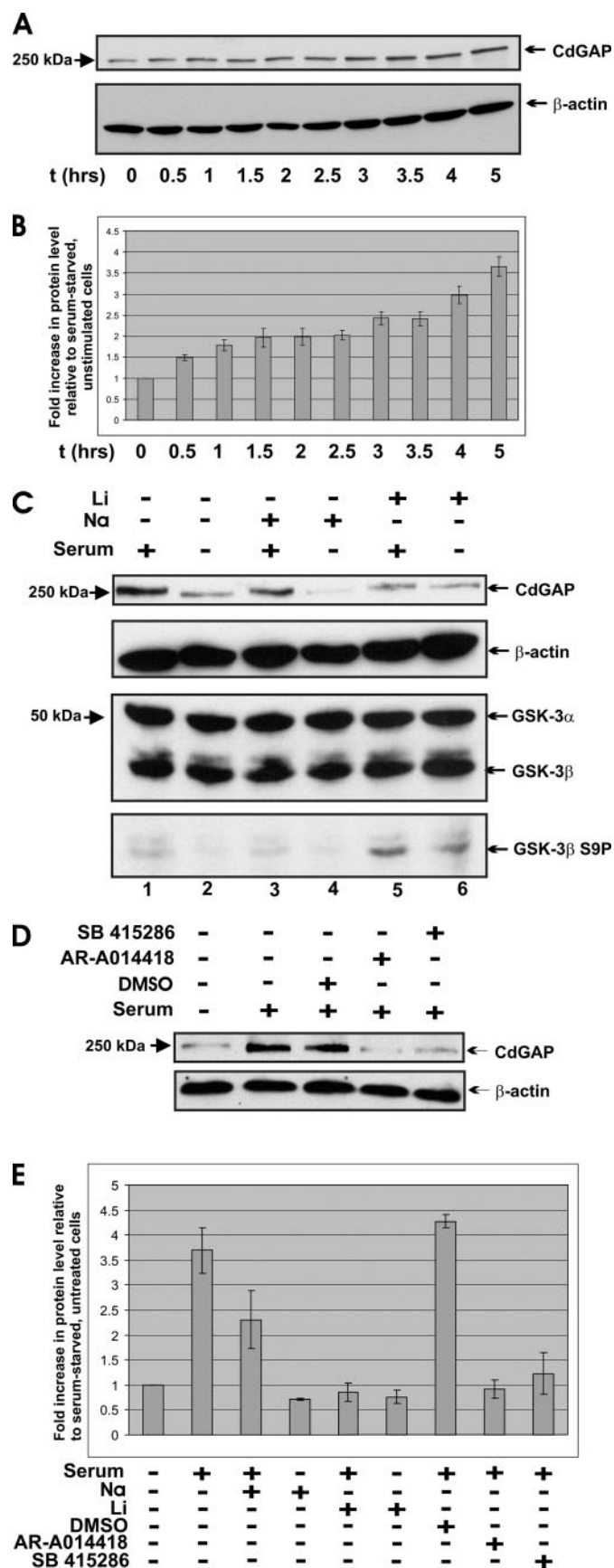


FIGURE 6. CdGAP protein levels are increased in response to serum in a GSK-3-dependent manner. *A*, NIH 3T3 cells were serum-starved overnight and were left unstimulated ( $t = 0$ ) or stimulated with 15% serum for the

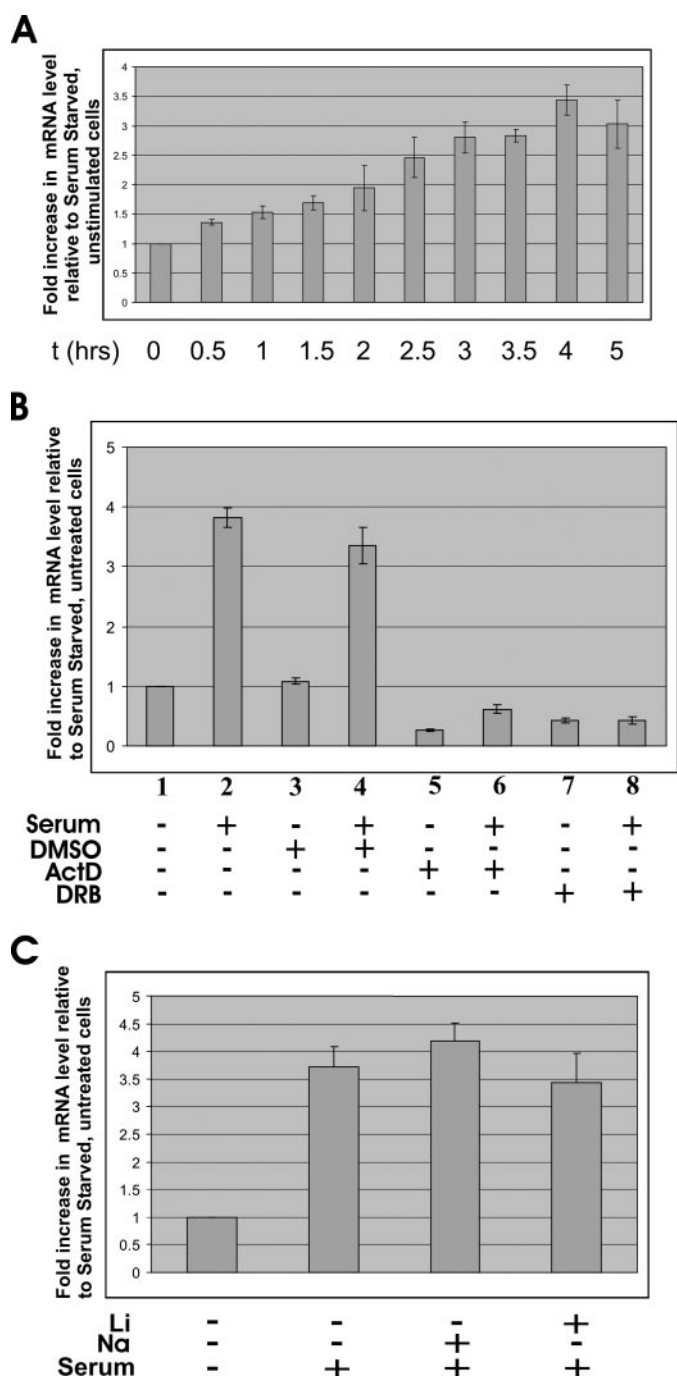
ulation and showed a 3.7-fold increase in CdGAP protein levels after 5 h of serum stimulation (Fig. 6*B*). It has been reported that GSK-3 is only transiently inhibited by growth factors such as epidermal growth factor and FGF-1, with GSK-3 regaining activity in as little as 20 min after stimulation (24, 25). Indeed, after 5 h of stimulation with serum, the levels of total GSK-3 were unchanged, and inactive GSK-3 phosphorylated on Ser-9 was barely detectable (Fig. 6*C*, lanes 1 and 2). Under these conditions, we found that inhibition of GSK-3 by LiCl did not alter the levels of CdGAP in serum-starved cells (Fig. 6*C*, compare lanes 2 and 6, and 6*E*); however, when GSK-3 activity was inhibited in cells stimulated with serum, the increase in CdGAP protein levels was blocked (Fig. 6*C*, compare lanes 1 and 5, and 6*E*). NaCl was used as a negative control and had a slight effect on the levels of CdGAP in either serum-stimulated or serum-starved cells (Fig. 6*C*, compare lanes 3 and 4 with lanes 1 and 2, and 6*E*). Consistent with the results obtained with LiCl inhibition of GSK-3, we found that both AR-A014418 and SB 415286 inhibited the increase in the levels of CdGAP proteins in response to serum (Fig. 6, *D* and *E*). Thus, these findings demonstrate that GSK-3 activity is necessary to regulate the levels of CdGAP proteins in response to serum.

To address whether the change in the levels of CdGAP protein resulted from a change in mRNA levels, we determined the mRNA levels of CdGAP in serum-stimulated NIH 3T3 fibroblasts by quantitative reverse transcription-PCR. As shown in Fig. 7*A*, the mRNA levels of CdGAP-1 increased over a period of 30 min to 5 h of serum stimulation, with a peak at 4 h (Fig. 7*A*). To determine whether this increase in CdGAP mRNA was mediated transcriptionally, serum-starved NIH 3T3 cells were pretreated with either ActD or DRB prior to serum stimulation. These compounds inhibit transcription of RNA (26). In serum-starved cells treated with ActD or DRB, the mRNA levels of CdGAP appeared to be reduced when compared with control cells (Fig. 7*B*, lanes 5 and 7). Following serum stimulation, a slight increase in the levels of CdGAP mRNA was observed in the presence of ActD, whereas there was no increase in CdGAP mRNA levels in DRB-treated cells (Fig. 7*B*, lanes 6 and 8). This demonstrates that transcription is involved in the process of up-regulation of mRNA levels; however, it does not exclude the possibility that there may be some alternative mechanisms, such as mRNA stabilization. To determine whether GSK-3 affects the levels of CdGAP mRNA, serum-starved cells were treated with LiCl or NaCl while being stimulated with serum. As shown in Fig. 7*C*, the mRNA levels of CdGAP showed a 4-fold increase following a 5-h stimulation with serum, similar

indicated times. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP and  $\beta$ -actin. *B*, quantitative analysis of *A* representing the amounts of CdGAP protein relative to those in serum-starved conditions. Error bars represent standard errors of the mean relative to three independent experiments. *C*, NIH 3T3 cells were serum-starved overnight and were left unstimulated or were stimulated for 5 h with 15% serum in the presence or absence of 50 mM NaCl or 50 mM LiCl. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP, phospho-Ser-9 GSK-3 $\beta$ , GSK-3 $\alpha$  and - $\beta$ , and  $\beta$ -actin. *D*, NIH 3T3 cells were serum-starved overnight and were left unstimulated or were stimulated for 5 h with 15% serum in the presence or absence of 0.1% Me<sub>2</sub>SO (DMSO) or 100  $\mu$ M AR-A014418 or 100  $\mu$ M SB 415286. Protein lysates were subjected to SDS-PAGE followed by Western blotting against CdGAP and  $\beta$ -actin. *E*, quantitative analysis of *C* and *D* was performed as above.



## GSK-3 Regulates CdGAP



**FIGURE 7. CdGAP mRNA levels are up-regulated by serum.** A, NIH 3T3 cells were serum-starved overnight and were left unstimulated ( $t = 0$ ) or were stimulated with 15% serum for the indicated times. Total RNA was extracted and submitted to reverse transcription followed by quantitative PCR to measure amounts of CdGAP and 18 S ribosomal subunit mRNAs. Quantitative analysis represents the amounts of CdGAP mRNA relative to those in serum-starved conditions. Error bars represent standard errors of the mean relative to at least three independent experiments. B, NIH 3T3 cells were serum-starved overnight and were then incubated with 0.1%  $\text{Me}_2\text{SO}$  (DMSO) (lanes 3 and 4), 10  $\mu\text{g/ml}$  ActD (lanes 5 and 6), or 100  $\mu\text{M}$  DRB (lanes 7 and 8) for 1 h before stimulation with 15% serum or 15% serum with  $\text{Me}_2\text{SO}$ , ActD, or DRB. Quantitative analysis of CdGAP mRNA levels was done as above. C, NIH 3T3 cells were serum-starved overnight and were left unstimulated or were stimulated for 5 h with 15% serum in the presence or absence of 50 mM NaCl or LiCl. Quantitative analysis of CdGAP mRNA levels was done as above.

to the increase in protein levels (Fig. 6B). Interestingly, the LiCl GSK-3 inhibitor did not inhibit the increase in CdGAP mRNA levels induced by serum (Fig. 7C). Taken together, these findings indicate that CdGAP expression is up-regulated by serum and that GSK-3 activity is necessary to regulate the levels of CdGAP proteins post-transcriptionally.

## DISCUSSION

To gain insight into the cellular function of CdGAP, we undertook a search for binding partners for the proline-rich domain of CdGAP, a region known to be important for the regulation of CdGAP activity (6). Through a yeast-two hybrid screen, we identified GSK-3 $\alpha$  as a binding partner for CdGAP-PRD and then determined that both GSK-3 $\alpha$  and GSK-3 $\beta$  are able to interact with the short and long isoforms of CdGAP in mammalian cells. This, coupled with our knowledge of CdGAP phosphorylation within the PRD, led us to examine whether CdGAP is a physiological substrate for GSK-3. We found that inhibiting GSK-3 activity greatly attenuated the phosphorylation of endogenous CdGAP in both NIH 3T3 (Fig. 3) and Swiss 3T3 fibroblasts (data not shown). Subsequent analysis *in vitro* led us to discover that GSK-3 can phosphorylate CdGAP on Thr-776 within the proline-rich domain of CdGAP. Using a polyclonal antibody that recognized the phosphorylated residue Thr-776 of CdGAP, we demonstrated that this residue is an *in vivo* target site of GSK-3 in both NIH 3T3 cells and U2OS osteosarcoma cells. The identification of Thr-776 as a GSK-3 phosphorylation site is of great interest for at least two reasons. First, this site is an atypical GSK-3 phosphorylation site. The motif  $^{776}\text{TPL}^{\text{EE}780}$  does not contain the usual +4 priming phospho-serine or phospho-threonine site that is typical for many GSK-3 substrates (21). Instead, it contains a negatively charged glutamic acid residue that can mimic the priming phosphorylation event required for the subsequent phosphorylation by GSK-3 (22). This may in part explain why GSK-3 efficiently phosphorylates recombinant CdGAP-PRD *in vitro* as it would not require this substrate to be primed by another kinase. Of note, however, is that amino acid substitution of Thr-776 to alanine did not completely eliminate GSK-3 phosphorylation of CdGAP *in vitro*, suggesting that other residues are phosphorylated by GSK-3. Second is the fact that this residue is an ERK 1 phosphorylation site important in the negative regulation of the GAP activity of CdGAP (8). Although there are not many examples of substrate sites that function for both ERK and GSK-3, it has been reported that the transcription factor CCAAT/enhancer binding protein  $\beta$ , and myelin basic protein (MBP) are phosphorylated at a consensus ERK/GSK-3 site (27, 28). In the case of CCAAT/enhancer binding protein  $\beta$ , it appears that its phosphorylation at a GSK-3/ERK consensus site is required for the induction of adiponectin gene expression during differentiation of mouse fibroblasts into adipocytes (27). For MBP, it is unclear what is the role of the consensus ERK/GSK-3 phosphorylation site, but this site is thought to play a role in changing the conformation of the protein, leading to changes in the interaction of MBP with the lipid bilayer in brain myelin (28). In the present study, our findings strongly suggest that phosphorylation of Thr-776 in the proline-rich domain of CdGAP is a consensus ERK-1/GSK-3 site required

for a tight regulation of CdGAP activity under different cellular conditions.

This study also demonstrates that the cellular protein levels of CdGAP are serum-responsive. Five hours after stimulation with serum, the protein levels of CdGAP within the cells are elevated, and we have shown that this is concomitant with an increase in the amount of CdGAP mRNA. The inhibition of CdGAP mRNA increase by ActD or DRB suggests that this process is transcriptionally mediated; however, it does not rule out the possibility that there may also be other mechanisms such as stabilization of the mRNA that contribute to this process as well. Interestingly, we found that GSK-3 activity is required for the serum-dependent increase in the cellular protein levels of CdGAP but not the mRNA levels. Thus, these findings indicate that GSK-3 is acting post-transcriptionally to regulate the protein levels of CdGAP. It remains to be determined whether GSK-3 stimulates CdGAP protein synthesis or regulates its stability. Although more studies have reported the converse situation, where GSK-3 phosphorylation leads to protein instability, there are a few examples of proteins being stabilized after phosphorylation by GSK-3, namely the retinoblastoma-related pocket protein RBL2/p130 (29), the nuclear receptor Rev-erb $\alpha$ , a negative component of the circadian clock (30), and axin, a component of the ternary complex including  $\beta$ -catenin and APC (31).

As reported earlier by many studies, RhoGAPs are regulated post-translationally via various molecular mechanisms such as lipid interaction (32), protein-protein interaction (6), phosphorylation (8, 33), and proteolytic degradation (34). Clearly, CdGAP utilizes at least two of these mechanisms of regulation, including phosphorylation (8) and protein-protein interactions (6) to control its GAP activity. Here, we report for the first time the regulation of a RhoGAP protein at the transcriptional level in response to serum. The induction in mRNA levels occurred early (1 h) and peaked at 4 h, suggesting that CdGAP expression may represent a novel mitogen-inducible early gene (35). Future studies will be required to determine the molecular pathways necessary to activate CdGAP gene expression and their consequences on cell proliferation, migration, and survival.

In conclusion, we have identified CdGAP as a novel GSK-3 substrate, and stimulation of the cellular protein and mRNA levels of CdGAP by serum provides a novel mechanism to control Cdc42/Rac1 GTPase signaling pathways.

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