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AGAP1, a Novel Binding Partner of Nitric Oxide-sensitive Guanylyl Cyclase*

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Nitric oxide (NO)-sensitive soluble guanylyl cyclase (sGC) is the major cytosolic receptor for NO, catalyzing the conversion of GTP to cGMP. In a search for proteins specifically interacting with human sGC, we have identified the multidomain protein AGAP1, the prototype of an ArfGAP protein with a GTPase-like domain, Ankyrin repeats, and a pleckstrin homology domain. AGAP1 binds through its carboxyl terminal portion to both the α_1 and β_1 subunits of sGC. We demonstrate that AGAP1 mRNA and protein are co-expressed with sGC in human, murine, and rat cells and tissues and that the two proteins interact *in vitro* and *in vivo*. We also show that AGAP1 is prone to tyrosine phosphorylation by Src-like kinases and that tyrosine phosphorylation potently increases the interaction between AGAP1 and sGC, indicating that complex formation is modulated by reversible phosphorylation. Our findings may hint to a potential role of AGAP1 in integrating signals from Arf, NO/cGMP, and tyrosine kinase signaling pathways.

Nitric oxide (NO)[1] is a potent mediator with pleiotropic functions such as inhibition of platelet activation, smooth muscle relaxation, vasodilatation, and regulation of neuronal transmission (1). These effects are mostly mediated by NO-sensitive soluble guanylyl cyclases (sGC) converting GTP into the second messenger, cGMP, which, in turn, regulates downstream effectors such as kinases, phosphodiesterases, and ion channels (2–4). Mammalian sGCs are obligate heterodimers consisting of an α and β subunit each (5–7), the most abundant isoform being $\alpha_1\beta_1$. Because sGC is one of the key regulators of intracellular cGMP levels, its activity is under tight control. On the translational level, sGC expression is down-regulated, *e.g.* in aging cells (8–10). On the protein level, allosteric activation via NO governs the activity of sGC. Also, homodimerization and heterodimerization may play a role in regulating sGC activity (11–13). The expression of alternatively spliced variants or isoforms of the β subunit may help modulate cellular cyclase activity through targeted degradation of sGC (14). An endogenous inhibitor of sGC has been isolated from bovine lung; however, the molecular identity of the 149-kDa protein is still unknown (15). Serine/threonine phosphorylation of sGC has also been demonstrated, yet the effects on sGC activity appear to be moderate (16–18). The binding of sGC to interacting proteins such as the scaffold protein PSD95 (19) or chaperone Hsp90 (20) may facilitate circumscribed cGMP production at or in cellular compartments. For instance binding of the $\alpha_2\beta_1$ isoform to PSD95 targets sGC to the postsynaptic complex in close proximity to neuronal NO synthase and cGMP-dependent effectors, thereby optimizing cGMP generation and signal propagation at the subsynaptic membrane (19). Although appealing, many of these hypotheses still await experimental proof *in vivo*.

ADP ribosylating factors (Arf) are a subfamily of GTP-binding proteins within the Ras superfamily and are involved in the regulation of membrane traffic and actin cytoskeleton dynamics (21, 22). The Arf proteins work as molecular switches (23), and their activity is regulated through the differential action of guanine nucleotide exchange factors and GTPase-activating proteins (GAPs). At least 16 distinct types of ArfGAPs are presently known that have been categorized in three major families, *i.e.* the ArfGAP1 type, the Git type, and the AZAP type (24). The multivalent scaffold protein AGAP1, also dubbed GGAP1 (25) or centaurin γ2 (26), is the prototype of the AGAP subfamily of AZAPs characterized by the presence of a GTP-binding protein-like domain (GLD), a split pleckstrin homology (PH) domain, the ArfGAP domain, and an ankyrin repeat domain (25, 27). AGAP1 has been shown to act as a phosphoinositide-dependent ArfGAP that impacts the endocytic compartment through the regulation of AP3-dependent trafficking (28) and affects the dynamics of the actin cytoskeleton (27).

In our search for novel proteins binding to NO-sensitive guanylyl cyclase we have identified AGAP1 as a novel and specific interaction partner of sGC *in vitro* and *in vivo*. We demonstrate that AGAP1 associates with both the α_1 and β_1 subunits of sGC and that complex formation between these proteins is modulated by tyrosine phosphorylation. Association with AGAP1 does not affect the enzymatic capacity of sGC nor does it alter its NO sensitivity. We propose that AGAP1, by binding to sGC, may help regulate the intracellular distribution of sGC and thus the local delivery of cGMP in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium and fetal calf serum were both obtained from PAA (Pasching, Austria); cell culture plasticware was from Greiner (Frickenhausen, Germany); ECL™ Western blotting detection reagents and glutathione-Sepharose™ 4B came from Amersham Biosciences; monoclonal antibodies to hemagglutinin (anti-

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[1] The abbreviations used are: NO, nitric oxide; AGAP, ArfGAP with a GLD, ankyrin repeats, and a PH domain; Arf, ADP ribosylating factor; ArfGAP, Arf GTPase-activating protein; AS, antiserum; DSS, disuccinimidyl suberate; E1–19, exons 1–19; GFP, green fluorescent protein; GLD, GTP-binding protein-like domain; GST, glutathione *S*-transferase; HA, hemagglutinin; hAGAP1, human AGAP1; mAGAP1, mouse AGAP1; PBS, phosphate-buffered saline; PH, pleckstrin homology; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; rAGAP1, rat AGAP1; sGC, NO-sensitive (soluble) guanylyl cyclase; VSV, vesicular stomatitis virus.

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HA.11) were purchased from Babco (Richmond, CA); monoclonal anti-GFP (clone B-2), anti-c-Src (clone H-12), and protein A/G PLUS-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the monoclonal antibody to phosphotyrosine anti-PY (Tyr(P)-100) was from Cell Signaling Technology (Beverly, MA); the human placenta MATCHMAKER cDNA library came from BD Biosciences; PP2 was bought from Calbiochem; and Complete protease inhibitor mixture was from Roche Applied Science. All other reagents including monoclonal anti-VSV (clone P5D4), monoclonal anti-GST (clone GST-2), disuccinimidyl suberate (DSS), dimethyl sulfoxide (Me_2SO), Na_3VO_4, phenylmethylsulfonyl fluoride, and aprotinin were purchased from Sigma.

Two-hybrid Assay—The plasmids pEG202 and pJG4-5 and the yeast strain EGY48 for the interaction trap assay, generously provided by Dr. Roger Brent (Massachusetts General Hospital, Boston, MA), were used as described previously (29). The cDNAs for the catalytic (cat) and regulatory (reg) domains of the human sGC α_1 and β_1 subunits, respectively, *i.e.* α_{1reg} (amino acid positions 1–419), α_{1cat} (466–690), β_{1reg} (1–348), and β_{1cat} (404–619), were amplified by PCR and subcloned into the pEG202 vector to create fusion proteins with the LexA DNA-binding domain. pEG202-α_{1cat} was introduced in EGY48 (MATα *trp1 his3 ura3 leu2*::6LexAop-*LEU2*) containing the reporter plasmid pSH18–34, and a human placenta MATCHMAKER cDNA library fused to the B42 activation domain was screened. Interactions were validated by growth and blue coloring on minimal agar plates lacking histidine, tryptophan, uracil, and leucine but containing 2% galactose, 1% raffinose, and 80 μg/ml X-gal, buffered at pH 7.

Antibody Production—Antibodies to the α_1 (AS587) and β_1 subunits (AS566) were raised in rabbits using peptide sequences unique to human sGC α_1 (positions 94–121) and β_1 (positions 593–614), respectively. Antibodies against the regulatory domain of human sGC α_1 (positions 1–419) or the catalytic domain of β_1 (positions 404–619) were raised by immunizing rabbits (AS558 and AS556, respectively) or mice (AS613, AS614) with the corresponding GST fusion proteins. Antibodies to human AGAP1 (hAGAP1) were raised in rabbits to peptides DER25 (positions 775–799; AS627) and SPK23 (460–482; AS625). The antibodies were affinity-purified using the respective peptides coupled to Affi-Gel-10 following the manufacturer's instructions (Bio-Rad).

Northern Blotting—A human multiple tissue 12-lane MTN™ blot was obtained from BD Biosciences, and mouse tissue NBA (normalized by amount of mRNA) blot came from BioChain (Hayward, CA). A cDNA probe covering nucleotide positions 19–287 of the coding sequence of hAGAP1 was ^{32}P-labeled by random priming using the Prime-It® II random primer labeling kit from Stratagene. Northern blots were incubated overnight at 68 °C with the radiolabeled cDNA probe in ExpressHyb™ solution (BD Biosciences).

Construction of Expression Plasmids—The cDNAs for the α_1 and β_1 subunits of human sGC were kindly provided by Dr. Harald Schmidt (University of Giessen, Germany). The coding region for α_1 was amplified by PCR and subcloned into the EcoRI-XhoI sites of pSG8, a modified version of the eukaryotic expression vector pSG5. The β_1 cDNA fragment was ligated into the EcoRI site of pEDmtxr. The cDNA clone encoding full-length hAGAP1 (GenBank™ accession numbers NP_055729 and NM_014914) was a generous gift of Dr. Takahiro Nagase (Kazusa DNA Research Institute, Chiba, Japan). Data bank searches identified the splice variants hAGAP1α (GenBank™ accession number BC060814) and hAGAP1β (GenBank™ accession number AK001722) as well as the mouse homologue mAGAP1 (GenBank™ accession numbers NP_835220 and NM_178119) and its corresponding gene. For transient expression of AGAP1 or the domains thereof, the corresponding cDNA segments were amplified by PCR and subcloned into pcDNA3.0 modified previously for the expression of N-terminally HA-tagged proteins. For the expression of GFP- or VSV-tagged AGAP1, the coding region was PCR amplified and subcloned into the pSG8 modified previously for in-frame expression of N-terminal tags or into pEGFP. The cDNA for wild type chicken Src (pSG5-Src) and the dominant-negative mutant thereof (pSG5-SrcK$^-$) were generous gifts of Dr. Rudi Busse (University of Frankfurt, Germany). All constructs were subjected to DNA sequencing prior to their use.

Transfections—COS-1 cells from African green monkey kidney were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections were done with DEAE-dextrane. In brief, a 10-cm dish containing 6×10^5 cells was washed with phosphate-buffered saline (PBS), and expression plasmids were applied in a 5.7-ml serum-free medium mixed with 300 μl of DEAE-dextrane (1 mg/ml) and 12 μl of chloroquine (50 mg/ml). After incubation for 2.5 h, cells were treated with 10% Me_2SO in PBS for 2 min and cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum for 30–48 h prior to use.

Immunoprecipitation and Western Blotting—A freshly prepared stock solution of pervanadate (10 mM Na_3VO_4 and 300 mM H_2O_2 incubated for 10 min at room temperature prior to use) was diluted in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Thirty to forty-eight hours after transfection the cells were incubated for 10–30 min at 37 °C in the absence or presence of 100 μM pervanadate (final concentration). To inhibit Src family tyrosine kinases, cells were incubated in the absence or presence of PP2 for 1 h at the indicated concentrations followed by incubation with 100 μM pervanadate for 30 min. Cells from a 60-mm dish were washed with ice-cold PBS and lysed for 20 min on ice with 0.4 ml of immunoprecipitation buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 40 mM β-glycerophosphate, 25 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride supplemented with complete protease inhibitor mixture). Cellular debris was pelleted at $20,000 \times g$ for 15 min, and the supernatant was incubated with the corresponding antisera for 2 h at 4 °C under rotation. Antibodies were precipitated with protein A/G PLUS-agarose. The samples were subjected to SDS-PAGE and Western blotting. Nitrocellulose membranes were blocked with 5% dry milk in PBS-T (PBS with 0.1% Tween 20) or, when using an antibody to phosphotyrosine, in 2% bovine serum albumin in PBS-T for 1 h at room temperature. Blots were incubated with primary antibody diluted in PBS-T and 5% dry milk or PBS-T with 2% BSA for 1 h, followed by a peroxidase-conjugated secondary antibody. Immunoreactive protein bands were detected by chemiluminescence using the ECL detection kit.

Tissue Preparation and Immunofluorescence Studies—For immunoprecipitation of AGAP1 or sGC subunits, anesthetized rats were killed, and the respective organs were excised, washed with PBS, and homogenized in immunoprecipitation buffer. After centrifugation ($20,000 \times g$ for 20 min), the supernatants were subjected to immunoprecipitation (see above). For immunofluorescence studies, COS-1 cells were transfected with constructs expressing HA-tagged hAGAP1 and sGC β_1. After 24 h cells were fixed with 2% paraformaldehyde and incubated with monoclonal anti-HA (Babco) and polyclonal anti-β_1 (Cayman Chemical, Ann Arbor, MI), followed by the secondary antibodies Cy3-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, respectively (Jackson ImmunoResearch, West Grove, PA). Labeled cells were analyzed with a Zeiss confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Cross-linking Studies—COS-1 cells were co-transfected with GFP- and VSV-tagged AGAP1. Thirty-six hours post-transfection, the cells were washed twice with PBS and resuspended in 20 mM HEPES, pH 7.4, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Cells were lysed by repeated freezing in liquid nitrogen and sonification followed by centrifugation at $20,000 \times g$ for 15 min. For cross-linking, DSS was added (final concentration 0.3 mM DSS and 5% Me_2SO), and the lysate was incubated for 30 min on ice. Vehicle alone (5% Me_2SO) was used as control. The reaction was stopped by adding Tris/HCl, pH 7.4 (final concentration 70 mM), and the samples were analyzed by SDS-PAGE and Western blotting using anti-VSV.

Sequence Analyses—Sequence comparisons and protein domain and genomic analyses were done with ClustalW, Prosite, Smart, Pfam, BLAST, and Evidence Viewer software, respectively.

RESULTS

Identification of a Novel sGC Interacting Protein—To identify proteins interacting with sGC, we employed the yeast two-hybrid interaction trap using the catalytic domain of the α_1 subunit (α_{1cat}; positions 466–690) as the bait and a human placenta cDNA library as the prey. In several rounds of screening, we identified a member of the centaurin family of ArfGAP proteins (30), AGAP1 (GGAP1, centaurinγ2, KIAA1099), as a potential binding partner of sGC. To extend these initial findings, we used four distinct baits derived from the α_1 and the β_1 subunits of sGC, *i.e.* the α_1 regulatory domain (α_{1reg}, positions 1–419), the α_1 catalytic domain (α_{1cat}, positions 466–690), the β_1 regulatory domain (β_{1reg}, 1–348), and the β_1 catalytic domain (β_{1cat}, positions 404–619). Employing the yeast mating assay, we found that the carboxyl terminal portion of AGAP1 (AGAP1$^{399-804}$) binds to both subunits of sGC. More specifically, AGAP1$^{399-804}$ binds to the catalytic domain of α_1 and to the regulatory domain of β_1 but not to the α_1 regulatory or β_1 catalytic domains (Fig. 1*A*).

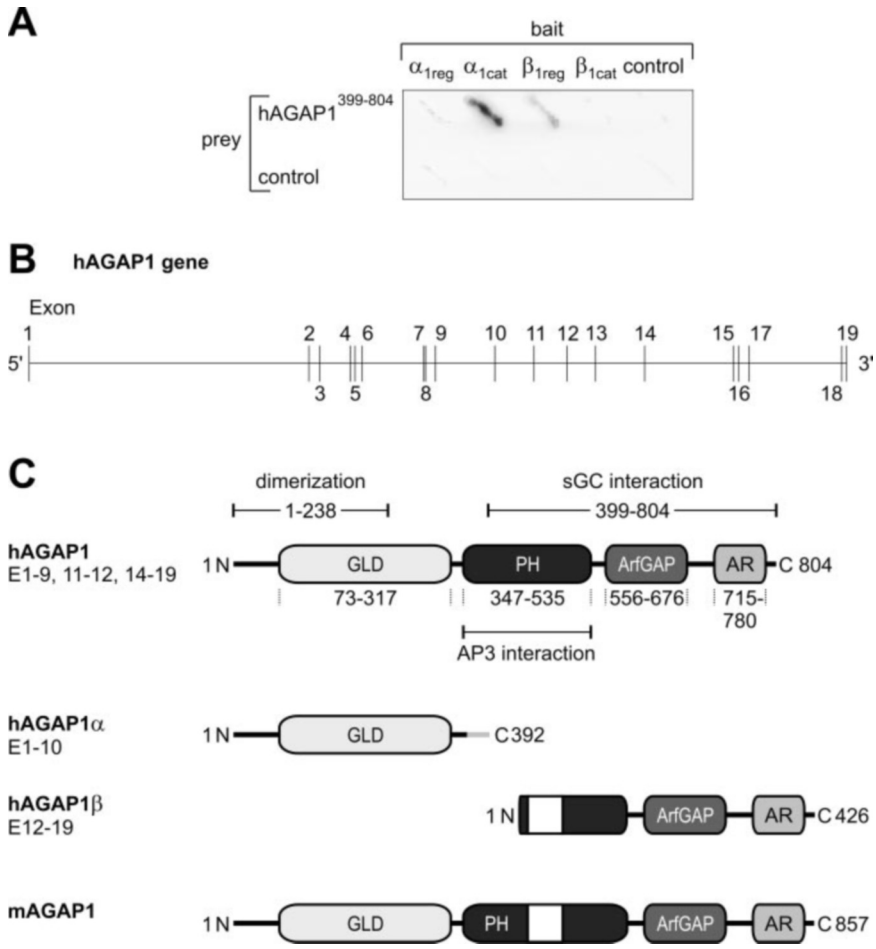

FIG. 1. **AGAP1 is a novel sGC-interacting protein.** *A*, the yeast mating system was employed using the catalytic (*cat*) and regulatory (*reg*) domains of the human sGC α_1 and β_1 subunits, respectively as baits, and hAGAP1$^{399-804}$ as the prey. *B*, structure of the human AGAP1 gene. The linear sequence of 19 exons (not drawn to scale) is shown. *C*, domain structure of mammalian AGAP1 variants. hAGAP1 of 804 residues comprises a single domain each of the GLD (positions 73–317), PH (347–535), ArfGAP (556–676), and ankyrin repeat type (*AR*; 715–780). The segments mediating dimerization, AP3 interaction, and sGC binding, respectively, are indicated. Two variants, *i.e.* hAGAP1α and hAGAP1β, most likely arising through alternative splicing of the primary transcript, are shown. mAGAP1 differs from hAGAP1 by an insertion of 53 residues (*white box*) in the split PH domain. The predicted exon arrangement of the corresponding mRNAs are shown below the names.

hAGAP1 is a multidomain protein of 804 amino acids with a calculated molecular mass of 89 kDa (27) comprising a single domain each of the GLD, PH, ArfGAP, and the ankyrin repeat type (Fig. 1*C*). AGAP1 is the product of a single gene of 19 exons on human chromosome 2, E1 through E19 (Fig. 1*B*). Through alternative splicing at least three products emerge, namely AGAP1 (E1–9, E11 and 12, and E14–19), AGAP1α (E1–10) (31) and AGAP1β (E12–19) (32). The mouse homolog, mAGAP1, is encoded by a gene of 19 exons located on mouse chromosome 1. The cloned mRNA retained exon 13, endowing mAGAP1 with an extra segment of 53 residues within the center part of the split PH domain, thus totaling 857 residues with a calculated molecular mass of 94 kDa (Fig. 1*C*). On the protein level, mAGAP1 and hAGAP1 have 91% sequence identity.

AGAP1 mRNA Expression Patterns—We investigated the expression patterns of the hAGAP1 and mAGAP1 genes by Northern blot analyses of various tissues and organs (Fig. 2, *A* and *B*). To this end, we constructed a ^{32}P-labeled cDNA probe covering nucleotide positions 19–287 of the coding sequence of hAGAP1. Transcripts of ~4.4 kbp were found for both messages, which is in reasonable agreement with their predicted sizes of 4078 bp (human) and 4234 bp (mouse). In human tissues, AGAP1 mRNA was present in the skeletal muscle, kidney, and placenta as well as in the brain, heart, colon, and lung (Fig. 2*A*). Low expression levels were found in the spleen, liver, and small intestine, whereas thymus and peripheral leukocytes failed to show significant AGAP1 mRNA levels. In the mouse, AGAP1 mRNA was prevalent in brain and kidney and present in heart, lung, spleen, and small intestine, whereas the level in skeletal muscle was below background (Fig. 2*B*). The distribution patterns observed for AGAP1 overlap largely with the known sGC α_1 and β_1 mRNA expression patterns in man and mouse (33, 34). In the brain, but also in skeletal muscle and the heart of both species, we found a strong additional signal of 2.4 kbp corresponding to the splice variant AGAP1α with a predicted size of 2184 bp (Fig. 2, *A* and *B*). Because of the

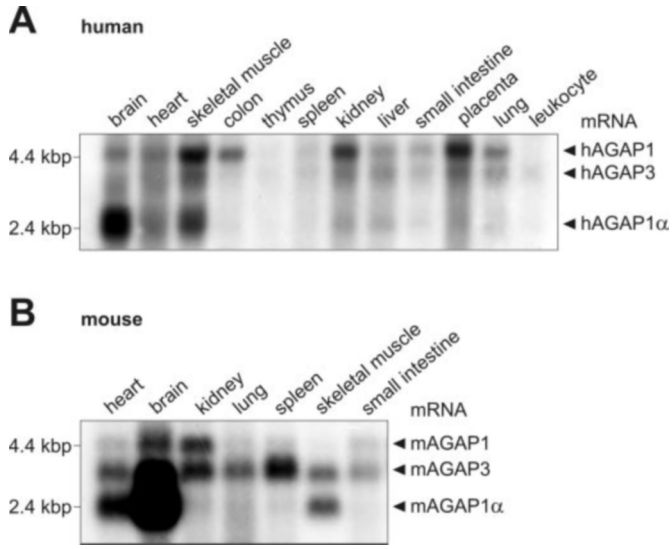

FIG. 2. **AGAP1 mRNA expression pattern.** Northern blot analyses of AGAP1 in human (*A*) and murine tissues (*B*). Equal amounts of human (1 μg per lane) and murine (3 μg per lane) poly(A)$^+$ mRNA were hybridized with a ^{32}P-labeled cDNA probe covering nucleotide positions 19–287 of the coding sequence of hAGAP1. Two major messages of 2.4 and 4.4 kbp are detected corresponding to AGAP1 and AGAP1α, respectively. Because of extensive sequence homology, the probe also recognized the message for AGAP3 (MRIP1 or centaurin γ3) of 3.2 kbp.

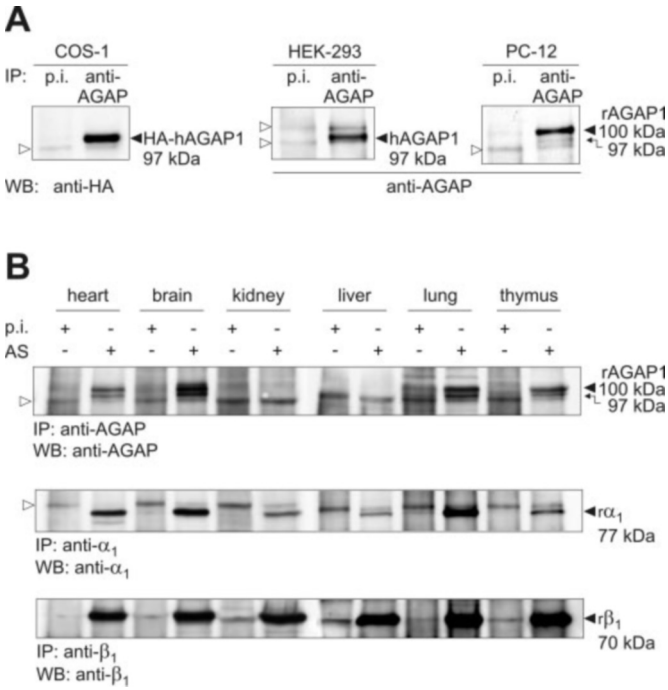

FIG. 3. **AGAP1 protein expression pattern.** *A*, immunoprecipitation (*IP*) from lysates of recombinant HA-tagged hAGAP1 transiently expressed in COS-1 cells, endogenous hAGAP1 from HEK-293 cells, and rAGAP1 from PC-12 cells using anti-AGAP (AS627). Western blotting (*WB*) was done with anti-HA (*left*) or anti-AGAP (AS625). For control, the corresponding pre-immune serum (*p.i.*) was used. The *small arrow* points to variant rAGAP1, and *open arrowheads* point to unspecific bands. The apparent molecular masses of the proteins are indicated on the *right*. *B*, immunoprecipitation of AGAP1 and sGC α_1 or β_1 from rat tissues using (from *top* to *bottom*) anti-AGAP (AS627), anti-α_1 (AS558), or anti-β_1 (AS556), respectively. Immunoprecipitates were analyzed by Western blotting (from *top* to *bottom*) with anti-AGAP (AS625), anti-α_1 (AS613) or anti-β_1 (AS614). Representatives of at least three independent experiments are shown.

high sequence identity among centaurins, our probe also detected a 3.2-kbp transcript of the AGAP3 gene (MRIP1, centaurin γ3), which is in perfect agreement with the predicted size of 3123 bp for the corresponding mRNA (GenBank™ accession number AF359283).

AGAP1 and sGC Protein Expression Patterns—We examined the expression of AGAP1 on the protein level (Fig. 3). To this end we produced the antisera AS625 and AS627 raised against synthetic peptides covering positions 460–482 and 775–799, respectively, of hAGAP1 and tested them in native and transfected cells of human, monkey, and rodent origin. AS627 immunoprecipitated recombinant HA-tagged hAGAP1 with an apparent molecular mass of 97 kDa from transiently transfected COS-1 cells, whereas the corresponding pre-immune serum failed to precipitate a band of similar size (Fig. 3*A*). Likewise, the antibody AS627 precipitated endogenous AGAP1 of 97 kDa from lysates of HEK-293 cells derived from human embryonic kidney (Fig. 3*A*) and from lysates of HeLa cells, but not from lysates of endothelium-derived EA.hy926 cells (not shown), demonstrating that these cells endogenously express AGAP1. Antiserum AS627 efficiently cross-reacted with the rat homologue rAGAP1 (100 kDa) in lysates of native PC-12 cells (Fig. 3*A*). In addition to the 100-kDa protein, which likely represents the full-length form of rAGAP1, AS627 immunoprecipitated a minor 97-kDa protein band that may represent the product of a differentially spliced mRNA in which the exon 13 not present in the human homolog has been skipped (Fig. 1*C*). Alternatively, the 97-kDa band may reflect a proteolytic breakdown product of rAGAP1; we have not tested these possibilities further. Using lysates from rat tissues, we immunoprecipitated rAGAP1 of 100 kDa (major) and 97 kDa (minor) from the heart, brain, lung, and thymus, whereas rAGAP1 was not detectable in the kidney and liver (Fig. 3*B*, *top*).

To monitor whether AGAP1 is co-expressed with its potential interaction partner, sGC, we used antisera raised against each of the sGC subunits and found both the α_1 (Fig. 3*B*, *center*) and the β_1 subunits (Fig. 3*B*, *bottom*) in the various rat organs. Thus, sGC and AGAP1 co-exist in several tissues and cells and, therefore, may interact *in vivo*.

Interaction of AGAP1 with sGC in Mammalian Cells—To demonstrate the interaction of hAGAP1 with sGC in mammalian cells, we used COS-1 cells co-transfected with constructs encoding HA-tagged AGAP1 and the α_1 and β_1 subunits of sGC, singly or combined. Western blots of the corresponding lysates using an anti-HA or a mixture of antibodies to the α_1 and β_1 subunits of sGC demonstrated that the various proteins were expressed at similar levels (Fig. 4*A*, *bottom*). Immunoprecipitation with two distinct anti-β_1 antisera followed by Western blotting with anti-HA revealed the association of HA-AGAP1 with sGC in lysates of triple transfected cells, whereas cells lacking sGC did not produce a significant band at 97 kDa (Fig. 4*A*, *top*). Failure of the corresponding pre-immune sera to precipitate a 97-kDa protein underlined the specificity of our system. Conversely, the antibody to HA-tagged AGAP1 coprecipitated the α_1 and β_1 subunits of sGC from lysates of triple transfected cells (not shown).

To extend our studies to endogenous proteins, we analyzed the interaction between AGAP1 and sGC in rat heart and brain, where both proteins are highly expressed (Fig. 3*B*). Immunoprecipitation with anti-α_1 or anti-β_1 followed by Western blotting with anti-AGAP demonstrated the association of AGAP1 with sGC in the rat heart (Fig. 4*B*, *top*) and brain (not shown). Furthermore, the presence of apparently equal amounts of α_1 and β_1 subunits in the precipitates suggested that anti-AGAP likely brought down intact heterodimeric sGC (Fig. 4*B*, *bottom*). The co-existence of sGC and AGAP1 was further demonstrated by immunofluorescence microscopy of intact COS-1 cells where the two proteins colocalized in the cytosol (Fig. 4*C*). Similar results were obtained with anti-α_1 (not shown). Hence, we conclude that multidomain protein AGAP1 may interact with sGC *in vitro* as well as *in vivo*.

In an initial effort to map the binding site(s) of AGAP1 for the sGC subunits, we used the yeast mating system and constructs of AGAP1 that cover the N-terminal portion of AGAP1 comprising a major part of GLD ($AGAP1^{1-238}$) or the C-terminal half of AGAP1 with PH, ArfGAP, and the ankyrin repeat domains ($AGAP1^{399-804}$) as negative and positive controls, respectively, as well as the isolated PH domain ($AGAP1^{347-535}$), the ArfGAP domain ($AGAP1^{556-678}$), and the ankyrin repeat domain ($AGAP1^{715-804}$) (Fig. 1*C*). Whereas $AGAP1^{1-238}$ was negative and $AGAP1^{399-804}$, *i.e.* the initially isolated construct, was positive for both the α_1 and β_1 subunits of sGC, none of the truncated versions of $AGAP1^{399-804}$ containing single PH, ArfGAP, or ankyrin repeat domains tested positive (not shown). These findings point to the possibility that the binding site(s) of AGAP1 for the sGC subunits may be discontinuous.

Homodimerization of AGAP1—In an effort to map the binding site we had initially used the LexA-based yeast two-hybrid system, which consistently indicated self-association of AGAP1 (not shown). To confirm these results in a mammalian cell line, we co-expressed two differentially tagged versions of AGAP1 where HA or GFP were fused to the N terminus of AGAP1 in COS-1 cells. Immunoprecipitation with anti-HA and Western blotting with anti-GFP revealed a strong band for GFP-AGAP1, indicative of its association with HA-AGAP1 (Fig. 5*A*). To de-

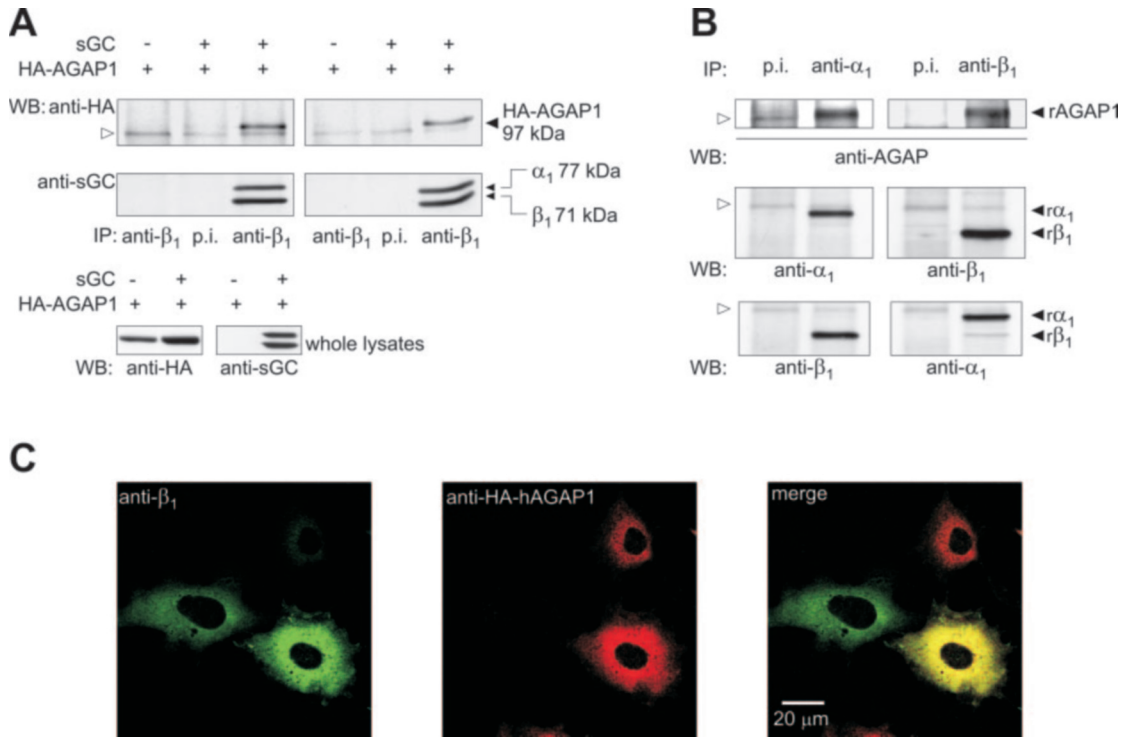

FIG. 4. **AGAP1 coprecipitates with sGC.** *A*, lysates from COS-1 cells overexpressing HA-tagged hAGAP1 and sGC were used for the immunoprecipitation (*IP*) of β_1 using two distinct anti-β_1 antisera, *i.e.* AS566 (*left*) and AS556 (*right*). Immunoprecipitates were analyzed by Western blotting (*WB*) with anti-HA (*top*) or a mixture of anti-α_1 (AS613) and anti-β_1 (AS614) (*bottom*). For control, the corresponding pre-immune (*p.i.*) sera were used. *Open arrowheads* point to unspecific bands. Representatives of at least three independent experiments are shown. *B*, immunoprecipitation of α_1 or β_1 from rat heart lysates using anti-α_1 (AS558) or anti-β_1 (AS556), respectively. Western blotting was performed using (from *top* to *bottom*) anti-AGAP (AS627), anti-α_1 (AS613), or anti-β_1 (AS614). Representatives of at least three independent experiments are shown. *C*, immunolocalization of sGC β_1 (*left*) and HA-hAGAP1 (*center*) in transfected COS-1 cells using confocal laser-scanning microscopy. *Right*, merged pictures.

fine more precisely the stoichiometry of this interaction, we cross-linked VSV-tagged AGAP1 of 97 kDa and GFP-AGAP1 of 120 kDa in whole lysates of COS-1 cells using the chemical cross-linker DSS. The resultant conjugates were analyzed by Western blotting with anti-VSV (Fig. 5*B*). In the absence of DSS, a single band representing the monomeric form of VSV-AGAP1 was seen, whereas cross-linking produced two additional bands of 190 and 210 kDa corresponding to the VSV-AGAP1 homodimer and the VSV-AGAP1/GFP-AGAP1 heterodimer, respectively. In a reblot with anti-GFP we confirmed the presence of the heterodimer, and at the same time we detected the monomeric and homodimeric forms of GFP-AGAP1 (not shown). Thus, it appears that at least a fraction of AGAP1 is present as a homodimer in a mammalian cell line.

To map the domains of hAGAP1 involved in dimerization, we co-expressed GFP-tagged AGAP1 and full-length HA-AGAP1 or shortened versions thereof in which single or multiple domains had been deleted (Fig. 5*C*). Full-length HA-AGAP1 coprecipitated with GFP-AGAP1, as did the fusion proteins of the N-terminal portion of AGAP1 containing (part of) the GLD domain, *i.e.* HA-AGAP1$^{1-238}$ and HA-AGAP1$^{1-345}$, respectively. By contrast, GFP-AGAP1 did not coprecipitate with the C-terminal portion of AGAP1$^{340-808}$ containing the PH, ArfGAP, and the ankyrin repeat domain (Fig. 5*D*). Together, these results suggest that the N-terminal portion of hAGAP1 (positions 1–238) comprising a truncated GLD domain mediates AGAP1 homodimerization and that this portion is distinctly different from the sGC-interacting domain in the C-terminal portion of AGAP1 (399–804). Thus, it appears that homodimerization of AGAP1 could serve to accommodate heterodimeric sGC.

Tyrosine Phosphorylation of AGAP1 by Src-like Kinases—Previous studies have demonstrated that ASAP1, *i.e.* an AGAP-like protein, is Tyr phosphorylated by Src (35). To test whether AGAP1 is also phosphorylated, we used COS-1 cells expressing HA-tagged hAGAP1 and incubated them with 100 μM of the broad spectrum protein tyrosine phosphatase inhibitor pervanadate. Following immunoprecipitation with anti-HA, Western blotting was done with anti-phosphotyrosine (Fig. 6*A*). In the absence of pervanadate, no significant Tyr phosphorylation of AGAP1 was observed. After 10 min of incubation with the inhibitor, a weak band appeared, and after 30 min a strong Tyr phosphorylation signal was evident for AGAP1. To probe for the role of Src-like kinases, we co-transfected COS-1 cells with wild type Src and a kinase-dead mutant, SrcK$^-$ (SrcK295M). Western blotting with anti-PY revealed a strong phosphorylation of AGAP1 by wild type Src but not with mutant SrcK$^-$ (Fig. 6*B*). To confirm these findings we pre-incubated cells expressing HA-AGAP1 with increasing concentrations of the Tyr kinase inhibitor PP2 targeted primarily to Src kinase family members (36, 37), followed by a 30-min incubation period with pervanadate and immunoprecipitation with anti-HA (Fig. 6*C*). PP2 efficiently prevented AGAP1 phosphorylation at 5 μM; the apparent IC_{50} was 0.5 μM. Reprobing the blots with anti-HA revealed that similar amounts of AGAP1 had been immunoprecipitated in each sample (Fig. 6*C*, *bottom*). Thus, Src-like kinase(s) likely mediate Tyr phosphorylation of AGAP1. Our initial screening for the target site(s) of Tyr kinases showed that both the N-terminal portion (AGAP1$^{1-345}$) and the C-terminal portion (AGAP1$^{538-804}$) were subject to Tyr phosphorylation, although to different degrees (Fig. 6*D*). Phenylalanine-scanning mutagenesis did not produce significantly reduced phosphorylation levels (not shown), suggesting that AGAP1 is indeed phosphorylated on multiple Tyr residues.

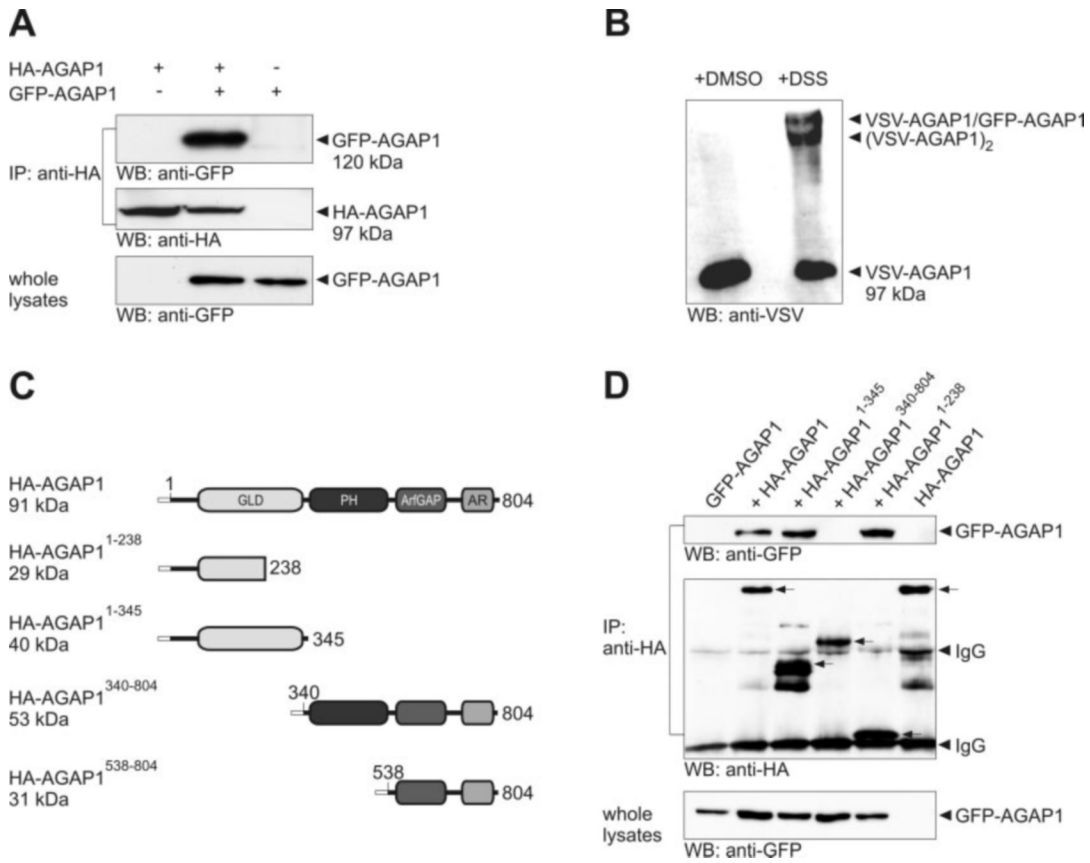

FIG. 5. **AGAP1 forms homodimers.** *A*, COS-1 cells were transiently transfected with HA-tagged or/and GFP-tagged hAGAP1 cDNAs. Immunoprecipitation (*IP*) was done with anti-HA, and Western blotting (*WB*) was accomplished with anti-HA or anti-GFP. *B*, COS-1 cells were co-transfected with hAGAP1 fused to GFP or VSV. Cross-linking was done with 0.3 mM DSS; for control, vehicle alone was used. The samples were analyzed by Western blotting using anti-VSV. *DMSO*, Me_2SO. *C*, constructs of hAGAP1 used for mapping. Numbers indicate the relative positions in the primary structure of hAGAP1; the calculated molecular masses of the constructs are given on the *left*. *D*, COS-1 cells overexpressing HA-tagged human AGAP1 and GFP-AGAP1 or shortened versions thereof were used for immunoprecipitation with anti-HA and Western blotting with anti-HA (*center*) or anti-GFP (*top* and *bottom*). *Arrows* point to the relevant bands identified on the *top*. Representatives of at least three independent experiments are shown.

Modulation of AGAP1-sGC Complex Formation by Tyrosine Phosphorylation—We asked for the functional consequences of Tyr phosphorylation for AGAP1 homodimerization and interaction with sGC. Even in the continuous presence of high concentrations of pervanadate we were unable to notice any effect of Tyr phosphorylation on the dimerizing capacity of AGAP1 (not shown). By contrast, Tyr phosphorylation had a marked impact on the complex formation between AGAP1 and sGC. Using COS-1 cells recombinantly expressing sGC and AGAP1, we could show that the interaction between the two proteins was significantly enhanced in the presence of pervanadate (Fig. 7*A*). Because AGAP1 interacts with both sGC subunits (Fig. 1*A*), we repeated the experiment in COS-1 cells co-expressing AGAP1 and one of each sGC subunit. Our immunoprecipitation experiments clearly revealed that the interactions of AGAP1 with both α_1 (Fig. 7*B*) and β_1 (Fig. 7*C*) were sensitive to pervanadate. These findings underline our initial conclusion that AGAP1 interacts with both sGC subunits and point to the fact that Tyr phosphorylation may critically modulate complex formation between AGAP1 and sGC.

DISCUSSION

In our quest for novel sGC-interacting proteins, we have identified the multidomain protein AGAP1 as a new binding partner for the $\alpha_1\beta_1$ isoform of sGC. AGAP1 is a member of the centaurin family of ArfGAP proteins, so named for their chimeric structure, which are involved in the regulation of membrane trafficking and/or actin cytoskeleton reorganization (26, 30). AGAP1 exposes ArfGAP activity toward Arf proteins in the order Arf1 > Arf5 > Arf6 (27, 28). Through binding to the δ and σ3 subunits of adaptor protein AP3, AGAP1 appears to regulate via Arf1 a specific endosomal compartment in mammalian cells (28). AP complexes are components of clathrin coats and mediate sorting events, *e.g.* at the *trans*-Golgi network and endosomes (38). AGAP1 also induces and localizes to endogenous AP1-containing structures, most likely endosomes, in NIH-3T3 cells (27). In this context our finding that AGAP1 interacts with sGC sheds new light on the recent observation that sGC copurifies with the μ subunit of AP1 from human platelet lysates (39). At present, the precise cellular localization of endogenous AGAP1 is unknown, largely because of the low affinity of the available antibodies (this study) (25, 27, 28). In overexpressing systems we and others have found a predominant cytosolic distribution in COS cells (this study) (25) and NIH-3T3 cells at high expression levels, whereas low expression levels resulted in vesicular staining for AGAP1 in these fibroblasts (27, 28). We have failed to observe a vesicular staining in COS-1 cells even at low AGAP1 expression; however, this may well reflect a cell type-specific phenomenon.

In general, proteins containing a PH domain(s) such as AGAP1 require membrane association for some aspects of their function, and they often bind with varying specificity and affinity to phosphoinositides embedded in membranes (40). Previous studies have shown that ArfGAP activity of AGAP1 is dependent on phosphatidylinositol phosphates such as phos-

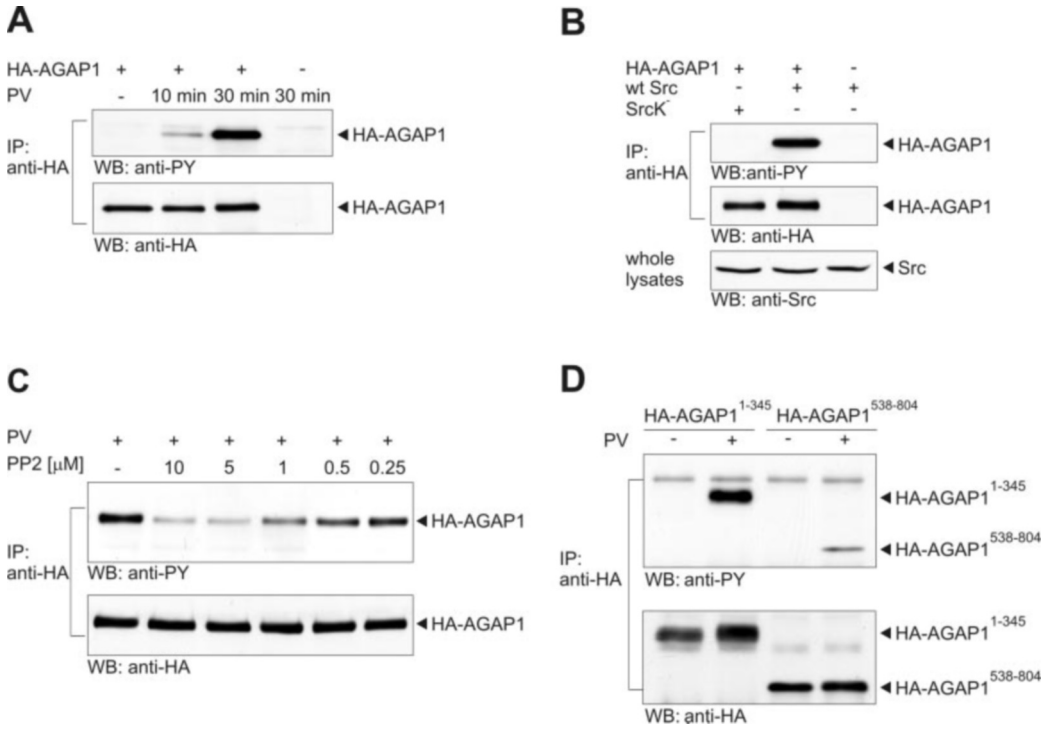

FIG. 6. **AGAP1 is phosphorylated by Src-like kinases.** *A*, COS-1 cells transfected with HA-tagged hAGAP1 were incubated for the indicated time intervals with 100 μM pervanadate (*PV*). Immunoprecipitation (*IP*) from cell lysates was done with anti-HA, and Western blotting (*WB*) was accomplished with anti-phosphotyrosine (*anti-PY*). *B*, COS-1 cells overexpressing HA-AGAP1 alone or in combination with wild type Src or kinase-dead mutant SrcK$^-$ were used. Immunoprecipitation and Western blotting were done as described above; phosphorylated AGAP1 was detected by anti-PY. *C*, COS-1 cells transfected with HA-AGAP1 were pre-incubated for 1 h with vehicle or PP2 at the indicated concentrations and exposed to 100 μM pervanadate for 30 min. Anti-HA was used for immunoprecipitation, and anti-PY or anti-HA was used for Western blotting. *D*, HA-AGAP1$^{1-345}$ or HA-AGAP1$^{538-804}$ was incubated in the presence (+) or absence (−) of 100 μM pervanadate for 30 min. HA-tagged proteins were immunoprecipitated from lysates by anti-HA, and proteins were detected in Western blots using anti-PY or anti-HA, as indicated. Representatives of at least three independent experiments are shown.

phatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-triphosphate (27). We have extended these studies and found that AGAP1, through its PH domain, binds to the phospholipids phosphatidylinositol 3-phosphate = phosphatidylinositol 4-phosphate = phosphatidylinositol 5-phosphate > phosphatidylinositol 3,4-bisphosphate > phosphatidylinositol 3,5-bisphosphate = phosphatidylinositol 4,5-bisphosphate = phosphatidylinositol 3,4,5-triphosphate.[2] Thus, AGAP1 may bind to phospholipids such as phosphatidylinositol 3-phosphate and anchor sGC at the plasma membrane, allowing the cyclase to increase local cGMP concentrations at specific cellular sites where it may serve distinct functions through cGMP-driven pathways. This notion is reinforced by the fact that cGMP-dependent pathways alter the cytoskeletal arrangement through cGMP-dependent protein kinases and their major substrate, *i.e.* vasodilator-stimulated phosphoprotein (VASP), ultimately causing the loss of VASP and zyxin from focal adhesions and promoting the disassembly of focal contacts (41).

To fulfill such hypothetical functions, the complex formation between AGAP1 and sGC must meet several criteria; *e.g.* AGAP1 should expose independent binding sites for AP complexes and sGC, its cyclase activity should be unimpeded by the complex formation, and this association should be governed by cellular signals. Our preliminary mapping studies indicate that AGAP1 binds sGC through its C-terminal portion comprising the ArfGAP and the ankyrin repeat domains, whereas the upstream PH domain is the primary docking site for phospholipids (this study) and for AP complexes such as AP3 (28). We have also considered the possibility that the association of sGC with AGAP1 may alter the enzymatic capacity of sGC; however, under the conditions of our experiments the cyclase activity was unchanged (data not shown). Finally, we have shown that AGAP1 is subject to phosphorylation by tyrosine kinases,

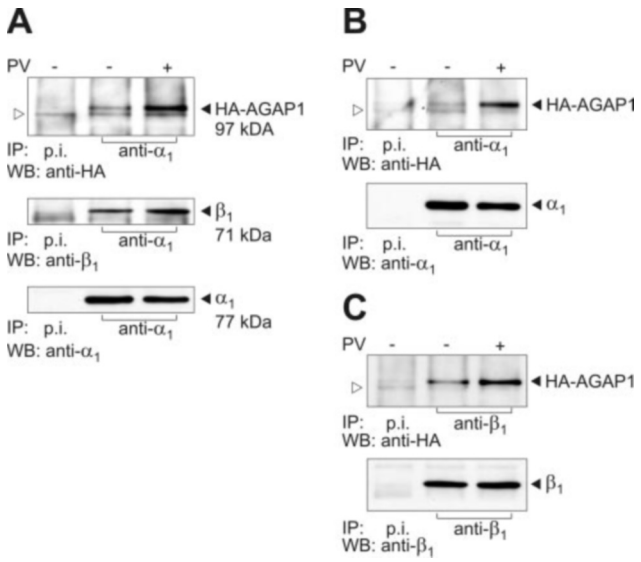

FIG. 7. **Tyr phosphorylation modulates interaction between AGAP1 and sGC.** COS-1 cells transfected with the cDNAs for HA-tagged hAGAP1 and α_1 and β_1 combined (*A*) or, individually, α_1 (*B*) and β_1 (*C*), were incubated in the absence (−) or presence (+) of 100 μM pervanadate for 10 min. Following lysis, immunoprecipitation (*IP*) was done with anti-α_1 (AS587; *A* and *B*) or anti-β_1 (AS566; *C*), and Western blotting (*WB*) was accomplished with anti-HA, anti-α_1 (AS587), or anti-β_1 (AS566) as indicated. *Open arrowheads* point to unspecific bands. Representatives of three independent experiments are shown. *p.i.*, pre-immune serum.

[2] S. Pioch, unpublished observations.

most likely by members of the Src family. Tyr phosphorylation of AGAP1 does not affect its dimerization capacity; however, it significantly enhances the interaction between AGAP1 and sGC, indicating that the complex formation between these proteins is subject to regulation by phosphorylation. Importantly, sGC is also Tyr phosphorylated under the conditions of our experiments.[3] Identification of the target sites and stimuli for Tyr phosphorylation as well as elucidation of the involved kinases and phosphatases will be instrumental to further investigate and finally comprehend the functional consequences of Tyr phosphorylation and sGC association with AGAP1.

An unexpected finding of this study is the homodimerization of AGAP1. The self-association of AGAP1 may have two important bearings. First, the homodimer of AGAP1 may more readily accommodate the heterodimeric sGC protein. Second, AGAP1 has been claimed to form an intramolecular complex through its N-terminal GTPase-like domain and its C-terminal ArfGAP domain, resulting in an enhanced GTPase activity that converts the enzyme to the inactive state (25). The finding that AGAP1 dimerizes may provide an alternative explanation, *i.e.* antiparallel binding of two monomers could favor the GDP-bound state through mutual interaction of the relevant domains. At present we do not know whether dimerization affects other important functions of AGAP1, nor do we know the functional consequences of sGC binding to AGAP1; we are currently investigating the potential implications of sGC binding for the ArfGAP activity.

To date, only two proteins have been reported to interact with the $\alpha_1\beta_1$ isoform of sGC, and both of them belong to the class of chaperon(in)s. The heat shock protein Hsp90 has been claimed to form a ternary complex with endothelial NO synthase and sGC (20). Although Hsp90 binding does not affect the basal activity of sGC, it enhances the NO donor-stimulated sGC activity by yet unknown mechanisms (20). Most recently, the η subunit of the chaperonin-containing *t*-complex polypeptide CCTη has been shown to bind sGC and inhibit the NO-stimulated activity of the wild type enzyme by 30–50% but not that of the constitutively active mutant $\alpha_1\beta_1^{Cys^{105}}$,where a crucial His^{105} residue has been changed to Cys (42). At the postsynaptic membrane the $\alpha_2\beta_1$ isoform of sGC interacts with PSD95 (19), which also binds to neuronal NO synthase. Both Hsp90 and CCTη bind to the β_1 subunit, whereas PSD95 binds to the α_2 subunit of sGC. Among the known sGC-binding proteins, AGAP1 stands out in that it binds to α_1 subunit; AGAP1 is also unique in that it serves as a bidental partner accommodating both subunits of sGC.

Although the functional implications of the many sGC interactions are not fully understood at this time, the following two notions are emerging. (i) Interaction with Hsp90 and PSD95 may help position sGC in juxtaposition to NO generators, optimizing cGMP production in membrane-proximal compartments; and (ii) binding to CCTη may serve to quickly desensitize the NO-stimulated cyclase. Our findings may point to yet another possibility, *i.e.* AGAP1 may position sGC in subcellular microdomains where it helps regulate endosomal trafficking and cytoskeletal rearrangements. In this respect, promiscuous binding may reflect the requirement for fine tuning on multiple levels to keep the activity and availability of a key enzyme such as sGC in check (43, 44). Indeed, our initial yeast two-hybrid screenings have identified yet another member of the centaurin subfamily of ArfGAP proteins (26, 30), namely MRIP2 (centaurinγ4), as a potential sGC binding protein. The overall sequence identity between AGAP1 and MRIP2 is 55% on the protein level, and the two centaurins share the domain structure except for GLD, which is lacking in MRIP2. Thus, it is quite possible that other centaurin(s) may contribute to intracellular sGC regulation.

In summary, our present study identifies a guanylyl cyclase-associated protein, AGAP1, which binds to the $\alpha_1\beta_1$ isoform of sGC in a phosphorylation-dependent manner. Our findings point to an unanticipated convergence of signal transduction pathways at the level of AGAP1 involving Arf-type GTPases, Src-like kinases, and the NO/cGMP signaling cascade.

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[3] S. Meurer, unpublished data.

REFERENCES

1. Bredt, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem.* **63,** 175–195
2. Münzel, T., Feil, R., Mülsch, A., Lohmann, S. M., Hofmann, F., and Walter, U. (2003) *Circulation* **108,** 2172–2183
3. Rybalkin, S. D., Yan, C., Bornfeldt, K. E., and Beavo, J. A. (2003) *Circ. Res.* **93,** 280–291
4. Barnstable, C. J., Wei, J. Y., and Han, M. H. (2004) *Neurochem. Int.* **45,** 875–884
5. Harteneck, C., Koesling, D., Söling, A., Schultz, G., and Böhme, E. (1990) *FEBS Lett.* **272,** 221–223
6. Buechler, W. A., Nakane, M., and Murad, F. (1991) *Biochem. Biophys. Res. Commun.* **174,** 351–357
7. Kamisaki, Y., Saheki, S., Nakane, M., Palmieri, J. A., Kuno, T., Chang, B. Y., Waldman, S. A., and Murad, F. (1986) *J. Biol. Chem.* **261,** 7236–7241
8. Bauersachs, J., Bouloumie, A., Mülsch, A., Wiemer, G., Fleming, I., and Busse, R. (1998) *Cardiovasc. Res.* **37,** 772–779
9. Chen, L., Daum, G., Fischer, J. W., Hawkins, S., Bochaton-Piallat, M. L., Gabbiani, G., and Clowes, A. W. (2000) *Circ. Res.* **86,** 520–525
10. Ruetten, H., Zabel, U., Linz, W., and Schmidt, H. H. (1999) *Circ. Res.* **85,** 534–541
11. Zabel, U., Häusler, C., Weeger, M., and Schmidt, H. H. (1999) *J. Biol. Chem.* **274,** 18149–18152
12. Gupta, G., Azam, M., Yang, L., and Danziger, R. S. (1997) *J. Clin. Investig.* **100,** 1488–1492
13. Gibb, B. J., Wykes, V., and Garthwaite, J. (2003) *Br. J. Pharmacol.* **139,** 1032–1040
14. Zhou, Z., Gross, S., Roussos, C., Meurer, S., Müller-Esterl, W., and Papapetropoulos, A. (2004) *J. Biol. Chem.* **279,** 24935–24943
15. Kim, T. D., and Burstyn, J. N. (1994) *J. Biol. Chem.* **269,** 15540–15545
16. Louis, J. C., Revel, M. O., and Zwiller, J. (1993) *Biochim. Biophys. Acta* **1177,** 299–306
17. Zwiller, J., Revel, M. O., and Malviya, A. N. (1985) *J. Biol. Chem.* **260,** 1350–1353
18. Zwiller, J., Revel, M. O., and Basset, P. (1981) *Biochem. Biophys. Res. Commun.* **101,** 1381–1387
19. Russwurm, M., Wittau, N., and Koesling, D. (2001) *J. Biol. Chem.* **276,** 44647–44652
20. Venema, R. C., Venema, V. J., Ju, H., Harris, M. B., Snead, C., Jilling, T., Dimitropoulou, C., Maragoudakis, M. E., and Catravas, J. D. (2003) *Am. J. Physiol.* **285**, H669–H678
21. Turner, C. E., West, K. A., and Brown, M. C. (2001) *Curr. Opin. Cell Biol.* **13,** 593–599
22. Moss, J., and Vaughan, M. (1998) *J. Biol. Chem.* **273,** 21431–21434
23. Vetter, I. R., and Wittinghofer, A. (2001) *Science* **294,** 1299–1304
24. Yoon, H. Y., Jacques, K., Nealon, B., Stauffer, S., Premont, R. T., and Randazzo, P. A. (2004) *Cell. Signal.* **16,** 1033–1044
25. Xia, C., Ma, W., Stafford, L. J., Liu, C., Gong, L., Martin, J. F., and Liu, M. (2003) *Mol. Cell. Biol.* **23,** 2476–2488
26. Jackson, T. R., Kearns, B. G., and Theibert, A. B. (2000) *Trends Biochem. Sci.* **25,** 489–495
27. Nie, Z., Stanley, K. T., Stauffer, S., Jacques, K. M., Hirsch, D. S., Takei, J., and Randazzo, P. A. (2002) *J. Biol. Chem.* **277,** 48965–48975
28. Nie, Z., Boehm, M., Boja, E. S., Vass, W. C., Bonifacino, J. S., Fales, H. M., and Randazzo, P. A. (2003) *Dev. Cell* **5,** 513–521
29. Dedio, J., König, P., Wohlfart, P., Schroeder, C., Kummer, W., and Müller-Esterl, W. (2001) *FASEB J.* **15,** 79–89
30. Hawadle, M. A., Folarin, N., Martin, R., and Jackson, T. R. (2002) *Biol. Res.* **35,** 247–265
31. Strausberg, R. L., Feingold, E. A., Grouse, L. H., Derge, J. G., Klausner, R. D., Collins, F. S., Wagner, L., Shenmen, C. M., Schuler, G. D., Altschul, S. F., Zeeberg, B., Buetow, K. H., Schaefer, C. F., Bhat, N. K., Hopkins, R. F., Jordan, H., Moore, T., Max, S. I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A. A., Rubin, G. M., Hong, L., Stapleton, M., Soares, M. B., Bonaldo, M. F., Casavant, T. L., Scheetz, T. E., Brownstein, M. J., Usdin, T. B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S. S., Loquellano, N. A., Peters, G. J., Abramson, R. D., Mullahy, S. J., Bosak, S. A., McEwan, P. J., McKernan, K. J., Malek, J. A., Gunaratne, P. H., Richards, S., Worley, K. C., Hale, S., Garcia, A. M., Gay, L. J., Hulyk, S. W., Villalon, D. K., Muzny, D. M., Sodergren, E. J., Lu, X., Gibbs, R. A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Young, A. C., Shevchenko, Y., Bouffard, G. G., Blakesley, R. W., Touchman, J. W., Green, E. D., Dickson, M. C., Rodriguez, A. C., Grimwood, J., Schmutz, J., Myers, R. M., Butterfield, Y. S., Krzywinski, M. I., Skalska, U., Smailus, D. E., Schnerch, A., Schein, J. E., Jones, S. J., and Marra, M. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 16899–16903

32. Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., Kimura, K., Makita, H., Sekine, M., Obayashi, M., Nishi, T., Shibahara, T., Tanaka, T., Ishii, S., Yamamoto, J., Saito, K., Kawai, Y., Isono, Y., Nakamura, Y., Nagahari, K., Murakami, K., Yasuda, T., Iwayanagi, T., Wagatsuma, M., Shiratori, A., Sudo, H., Hosoiri, T., Kaku, Y., Kodaira, H., Kondo, H., Sugawara, M., Takahashi, M., Kanda, K., Yokoi, T., Furuya, T., Kikkawa, E., Omura, Y., Abe, K., Kamihara, K., Katsuta, N., Sato, K., Tanikawa, M., Yamazaki, M., Ninomiya, K., Ishibashi, T., Yamashita, H., Murakawa, K., Fujimori, K., Tanai, H., Kimata, M., Watanabe, M., Hiraoka, S., Chiba, Y., Ishida, S., Ono, Y., Takiguchi, S., Watanabe, S., Yosida, M., Hotuta, T., Kusano, J., Kanehori, K., Takahashi-Fujii, A., Hara, H., Tanase, T. O., Nomura, Y., Togiya, S., Komai, F., Hara, R., Takeuchi, K., Arita, M., Imose, N., Musashino, K., Yuuki, H., Oshima, A., Sasaki, N., Aotsuka, S., Yoshikawa, Y., Matsunawa, H., Ichihara, T., Shiohata, N., Sano, S., Moriya, S., Momiyama, H., Satoh, N., Takami, S., Terashima, Y., Suzuki, O., Nakagawa, S., Senoh, A., Mizoguchi, H., Goto, Y., Shimizu, F., Wakebe, H., Hishigaki, H., Watanabe, T., Sugiyama, A., Takemoto, M., Kawakami, B., Watanabe, K., Kumagai, A., Itakura, S., Fukuzumi, Y., Fujimori, Y., Komiyama, M., Tashiro, H., Tanigami, A., Fujiwara, T., Ono, T., Yamada, K., Fujii, Y., Ozaki, K., Hirao, M., Ohmori, Y., Kawabata, A., Hikiji, T., Kobatake, N., Inagaki, H., Ikema, Y., Okamoto, S., Okitani, R., Kawakami, T., Noguchi, S., Itoh, T., Shigeta, K., Senba, T., Matsumura, K., Nakajima, Y., Mizuno, T., Morinaga, M., Sasaki, M., Togashi, T., Oyama, M., Hata, H., Komatsu, T., Mizushima-Sugano, J., Satoh, T., Shirai, Y., Takahashi, Y., Nakagawa, K., Okumura, K., Nagase, T., Nomura, N., Kikuchi, H., Masuho, Y., Yamashita, R., Nakai, K., Yada, T., Ohara, O., Isogai, T., and Sugano, S. (2004) *Nat. Genet.* **36,** 40–45
33. Budworth, J., Meillerais, S., Charles, I., and Powell, K. (1999) *Biochem. Biophys. Res. Commun.* **263,** 696–701
34. Mergia, E., Russwurm, M., Zoidl, G., and Koesling, D. (2003) *Cell. Signal.* **15,** 189–195
35. Brown, M. T., Andrade, J., Radhakrishna, H., Donaldson, J. G., Cooper, J. A., and Randazzo, P. A. (1998) *Mol. Cell. Biol.* **18,** 7038–7051
36. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) *Biochem. J.* **371,** 199–204
37. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) *J. Biol. Chem.* **271,** 695–701
38. Crottet, P., Meyer, D. M., Rohrer, J., and Spiess, M. (2002) *Mol. Biol. Cell* **13,** 3672–3682
39. Koglin, M., and Behrends, S. (2004) *Biochem. Pharmacol.* **67,** 1579–1585
40. Cullen, P. J., Cozier, G. E., Banting, G., and Mellor, H. (2001) *Curr. Biol.* **11,** R882–R893
41. Smolenski, A., Poller, W., Walter, U., and Lohmann, S. M. (2000) *J. Biol. Chem.* **275,** 25723–25732
42. Hanafy, K. A., Martin, E., and Murad, F. (September 14, 2004) *J. Biol. Chem.* DOI 10.1074/jbc.M404134200
43. Nedvetsky, P. I., Sessa, W. C., and Schmidt, H. H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 16510–16512
44. Tonks, N. K., and Neel, B. G. (2001) *Curr. Opin. Cell Biol.* **13,** 182–195