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

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Nitrergic Oxidase 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 α1 and β1 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.

Nitrergic Oxidase NO1 is a potent mediator with pleiotropic functions such as inhibition of platelet activation, smooth muscle relaxation, vasodilatation, and regulation of neuronal transmission. 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 α1β1. 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.

1 The abbreviations used are: NO, nitric oxide; AGAP, ArfGAP with a GTPase-like domain, ankyrin repeats, and a pleckstrin homology domain; Arf, ADP ribosylating factors; 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.

2 (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 α1 and β1 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.

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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.

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 ArfGAP 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 α1 and β1 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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HA.11) were purchased from Babco (Richmond, CA); mononclonal anti-
GFP (clone B-2), and protein A/G PLUS-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); the monoclonal anti-phospho-p44/42 mitogen-activated protein kinase antibody was from Cell Signaling Technology (Beverly, MA); the human placenta MATCH-
MAKER 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-BSV
(clone 5D4), monoclonal anti-GST (clone GST-2), disuccinimidyl su-
berate (DSS), dimethyl sulfoxide (Me2SO), Na3VO4, phenylmethylsul-
fonyl fluoride, and ATP were purchased from Sigma.

Two-hybrid Assays—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 α1 and β1 subunits, respectively, i.e., α1cat (amino acid positions 1–419), α1reg (positions 404–619) were raised by immunizing rabbits (AS558 and AS556, respectively) or mice (AS613, AS614) with the respective GST fusion proteins. 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 MTNTM 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 32P-labeled by random priming using the Prime-It® II random primer labeling kit from Stratagene. Northern blots were incu-
bated overnight at 68 °C with the radiolabeled cDNA probe in Ex-
pressHybTM solution (BD Biosciences).

Construction of Expression Plasmids—The cDNAs for the α1 and β1 subunits of human sGC were kindly provided by Dr. Harold Schmidt (University of Giessen, Germany). The coding region for α1was ampli-
fied by PCR and subcloned into the EcoRI-XhoI sites of pSG5, a modi-
fied version of the eukaryotic expression vector pSG8, a modified previously for in-frame expression of N-terminal tags or into the yeast expres-
sion vector pJG4-5 (29) (GenBankTM accession numbers NP_055729 and NM_014914) was a generous gift of Dr. Takahiro Na-
dome. pEG202-α1cat, introduced into yeast strain EGY48 for the interaction trap assay, was amplified by PCR and subcloned into the pEG202 vector to create fusion proteins with the LexA DNA-binding domain. pEG202-α1cat was introduced in EGY48 (MATa trpl1 his3 ura3 leu2::LexAop-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 morphological 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 α1 (AS587) and β1 subunits (AS566) were raised in rabbits using peptide sequences unique to hu-
man sGC α1 (positions 94–121) and β1 (positions 593–614), respec-
ively. Antibodies against the regulatory domain of human sGC α1 (positions 1–419) or the catalytic domain of human sGC β1 (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 to peptides coupled to Affi-
Gel-10 following the manufacturer’s instructions (Bio-Rad).

AGAP1 Interacts with Guanylyl Cyclase

Immunoprecipitation and Western Blotting—A freshly prepared stock solution of pervanadate (10 mM Na3VO4 and 300 mM H2O2, incubated for 10 min at room temperature prior to use) was diluted in modified Dulbecco’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 Na3P2O7, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride supplemented with complete protease inhibitor mixture). Cellular debris was pelleted at 20,000 × g for 15 min, and the supernatant was incubated with the corresponding anti sera for 2 h at 4 °C under rotation. Antibodies were preincubated with protein A/G PLUS-agarose. The samples were sub-
jected 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 phosphorysorinosine, in 2% bovine serum albu-
mun 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. Immuno reactive protein bands were detected by chemiluminescence using the ECL detection kit (Amersham).

Tissue Preparation and Immunofluorescence Studies—For immuno-
precipitation of AGAP1 or sGC subunits, anesthetized rats were killed, and the respective organs were excised, washed with PBS, and homog-
emized in immunoprecipitation buffer. After centrifugation (20,000 × g for 20 min), the supernatant were subjected to immunoprecipitation
(see below). For immunofluorescence, GFP- or VSV-tagged AGAP1 were expressed in 3T3 cells transiently transfected with constructs expressing HA-tagged hAGAP1 and sGC β1. After 24 h cells were fixed with 2% paraformaldehyde and incubated with monoclonal anti-HA (Babo) and polyclonal anti-β1 (Cayman Chemical, Ann Arbor, MI), followed by the secondary antibodies Cy3-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conju-
gated 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 ml HEPES, pH 7.4, 10 mM MnCl2, and 1 mM dithiothreitol. Cells were lysed by repeated freezing in liquid nitrogen and sonication followed by centrifugation at 14,000 × g for 15 min. For cross-linking, GFP- or VSV-tagged AGAP1 cells were transiently transfected with glutathione beads (final concentration, 0.3 mM DSS and 5% DSS, and the lysate was incubated for 30 min on ice. Vehicle alone (5% Me2SO) 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 α1 subunit (α1cat, 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, centaurin-[y2, KIAA1099], as a potential binding partner of sGC. To extend these initial find-
ings, we used four distinct bait derived from the α1 and the β1 subunits of sGC, i.e. the α1 regulatory domain (α1reg, positions 1–419), the α1 catalytic domain (α1cat, positions 466–690), the β1 regulatory domain (β1reg, positions 1–348), and the β1 catalytic domain (β1cat, positions 404–619). Employing the yeast mating assay, we found that the carboxyl terminal portion of AGAP1 (AGAP1399–804) binds to both subunits of sGC. More specifically, AGAP1399–804 binds to the catalytic domain of α1 and to the regulatory domain of β1, but not to the α1 regulatory or β1 catalytic domains (Fig. 1A).

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
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 hAGAP1 (E1–9, E11 and 12, and E14–19), hAGAP1 (E1–10) (31) and hAGAP1 (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 32P-labeled cDNA probe covering nucleotide positions 19–287 of the coding sequence of hAGAP1. Transcripts of 4.4 kbp were found for both mRNAs, 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 and β subunit 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
AGAP1 Interacts with Guanylyl Cyclase

49349

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 α1 or β1 from rat tissues using (from top to bottom) anti-AGAP (AS627), anti-α1 (AS558), or anti-β1 (AS556), respectively. Immunoprecipitates were analyzed by Western blotting (top to bottom) with anti-AGAP (AS625), anti-α1 (AS613) or anti-β1 (AS614). Representatives of at least three independent experiments are shown.

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. 3A). Likewise, the antibody AS627 precipitated endogenous AGAP1 of 97 kDa from lysates of HEK-293 cells derived from human embryonic kidney (Fig. 3A) 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. 3A). 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. 1C). 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. 3B, 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 α1 (Fig. 3B, center) and the β1 subunits (Fig. 3B, 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 α1 and β1 subunits of sGC, singly or combined. Western blots of the corresponding lysates using anti-HA or a mixture of antibodies to the α1 and β1 subunits of sGC demonstrated that the various proteins were expressed at similar levels (Fig. 4A, bottom). Immunoprecipitation with two distinct anti-β1 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. 4A, 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 α1 and β1 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. 3B). Immunoprecipitation with anti-α1 or anti-β1 followed by Western blotting with anti-AGAP demonstrated the association of AGAP1 with sGC in the rat heart (Fig. 4B, top) and brain (not shown). Furthermore, the presence of apparently equal amounts of α1 and β1 subunits in the precipitates suggested that anti-AGAP likely brought down intact heterodimeric sGC (Fig. 4B, 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. 4C). Similar results were obtained with anti-α1 (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 (AGAP11–238) or the C-terminal half of AGAP1 with PH, ArfGAP, and the ankyrin repeat domains (AGAP1199–804) as negative and positive controls, respectively, as well as the isolated PH domain (AGAP1347–535), the ArfGAP domain (AGAP1556–678), and the ankyrin repeat domain (AGAP1715–804) (Fig. 1C). Whereas AGAP11–238 was negative and AGAP1199–804, i.e., the initially isolated construct, was positive for both the α1 and β1 subunits of sGC, none of the truncated versions of AGAP1199–804 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. 5A). To de-
AGAP1 Interacts with Guanylyl Cyclase

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 β1 using two distinct anti-β1 antisera, i.e. AS566 (left) and AS556 (right). Immunoprecipitates were analyzed by Western blotting (WB) with anti-HA (top) or a mixture of anti-α1 (AS613) and anti-β1 (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 α1 or β1 from rat heart lysates using anti-α1 (AS558) or anti-β1 (AS556), respectively. Western blotting was performed using (from top to bottom) anti-AGAP (AS627), anti-α1 (AS613), or anti-β1 (AS614). Representatives of at least three independent experiments are shown. C, immunolocalization of sGC β1 (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. 5B). 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. 5C). 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-AGAP11–238 and HA-AGAP11–345, respectively. By contrast, GFP-AGAP1 did not coprecipitate with the C-terminal portion of AGAP1340–808 containing the PH, ArfGAP, and the ankyrin repeat domain (Fig. 5D). 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. 6A). 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. 6B). 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. 6C). PP2 efficiently prevented AGAP1 phosphorylation at 5 μM; the apparent IC50 was 0.5 μM. Repробing the blots with anti-HA revealed that similar amounts of AGAP1 had been immunoprecipitated in each sample (Fig. 6C, 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 (AGAP11–345) and the C-terminal portion (AGAP1358–804) were subject to Tyr phosphorylation, although to different degrees (Fig. 6D). Phenylalanine-scanning mutagenesis did not produce significantly reduced phosphorylation levels (not shown), suggesting that AGAP1 is indeed phosphorylated on multiple Tyr residues.
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. 7A). Because AGAP1 interacts with both sGC subunits (Fig. 1A), 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 α1 and α3 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 α3 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-
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. 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.

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.\textsuperscript{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 bearing. 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 αβ₂ isofrom 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 chaperon-containing t-complex polyepideptide 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 αβ₂(Cys\textsuperscript{105}→Ser)\textsuperscript{105}, where a crucial His\textsuperscript{105} residue has been changed to Cys (42). At the posttransmembate the αβ₂ isofrom of sGC interacts with PSD95 (19), which also binds to neuronal NO synthase. Both Hsp90 and Cctη bind to the β₁ subunit, whereas PSD95 binds to the α₂ subunit of sGC. Among the known sGC-binding proteins, AGAP1 stands out in that it binds to α₂ 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 (19), which also binds to neuronal NO synthase. Both Hsp90 and Cctη bind to the β₁ subunit, whereas PSD95 binds to the α₂ subunit of sGC. Among the known sGC-binding proteins, AGAP1 stands out in that it binds to α₂ 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 screening has identified yet another member of the centaurin subfamily of ArfGAP proteins (26, 30), namely MRIP2 (centaurin-y4), 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 struc-

\textsuperscript{3} S. Meurer, unpublished data.
AGAP1 Interacts with Guanylyl Cyclase

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