Identification of a receptor-independent activator of G protein signaling (AGS8) in ischemic heart and its interaction with Gβγ

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Edited by Lutz Birnbaumer, National Institutes of Health, Research Triangle Park, NC, and approved November 18, 2005 (received for review August 26, 2005)

As part of a broader effort to identify postreceptor signal regulators involved in specific diseases or organ adaptation, we used an expression cloning system in Saccharomyces cerevisiae to screen cDNA libraries from rat ischemic myocardium, human heart, and a prostate leiomyosarcoma for entities that activated G protein signaling in the absence of a G protein coupled receptor. We report the characterization of activator of G protein signaling (AGS) 8 (KIAA1866), isolated from a rat heart model of repetitive transient ischemia. AGS8 mRNA was induced in response to ventricular ischemia but not by tachycardia, hypertrophy, or failure. Hypoxia induced AGS8 mRNA in isolated adult ventricular cardiomyocytes but not in rat aortic smooth muscle cells, endothelial cells, or cardiac fibroblasts, suggesting a myocyte-specific adaptation mechanism involving remodeling of G protein signaling pathways. The bioactivity of AGS8 in the yeast-based assay was independent of guanine nucleotide exchange by Go, suggesting an impact on subunit interactions. Subsequent studies indicated that AGS8 interacts directly with Gβγ and this occurs in a manner that apparently does not alter the regulation of the effector PLC-β2 by Gβγ. Mechanistically, AGS8 appears to promote G protein signaling by a previously unrecognized mechanism that involves direct interaction with Gβγ.

accessory protein | signal adaptation | hypoxia

Signal processing via heterotrimeric G proteins (Goβγ) is one of the most widely used systems for information transfer across the cell membrane. Dysfunctional processing of signals through these transducing systems results in organ maladaptation and is characteristic of various disease states. In addition to G protein-coupled receptor (GPCR)-mediated activation of G protein signaling, nature has evolved other modes of signal input to G proteins that either act independent of a GPCR or modulate signal transfer from receptor to G protein. Recent studies also indicate a role of Go and Gβγ in the processing of signals within the cell distinct from those that mediate following activation by a cell-surface GPCRs (1–10). In such situations, the G protein subunits (Go and Gβγ) may actually be complexed with alternative binding partners independent of the typical heterotrimeric Goβγ.

A variety of approaches (protein interactions strategies, genetic screens, functional screens) have led to the identification of several nonreceptor proteins that indeed influence G protein signaling. Such proteins include the family of RGS proteins that accelerate the GTPase activity of Go and various entities that influence nucleotide binding properties and/or subunit interactions (2). The latter group of proteins includes activator of G protein signaling (AGS) 1–4, which were identified in a yeast-based functional screen for receptor-independent activators of G protein signaling as an extension of earlier biochemical characterization of such bioactivity in cell and tissue extracts (1–3, 11–13). The interaction of AGS1, AGS3, and AGS3-related proteins with heterotrimeric G proteins has subsequently been shown to regulate a diverse set of functions including asymmetric cell division, addictive behavior, and the circadian rhythm (1, 3–7, 14–18).

Such regulatory mechanisms are of particular interest because they may allow subtle alterations in signal processing as required by the cell as it meets various challenges placed upon it by alterations in its microenvironment. At the same time, such regulatory proteins may also be involved in the disease process itself by participating in the resetting of signaling set points as tissues “overcompensate” in various situations. As part of a broader approach to define signal regulators involved in the remodeling of G protein signaling networks that occurs in response to physiological or pathophysiological challenges, we used a functional screen to identify receptor-independent AGSs up-regulated in the face of such a challenge.

Here, we report the identification of a hypoxia-inducible, receptor-independent AGS (AGS8) that is induced as a part of cardiomyocyte adaptation to an ischemic challenge. AGS8 is a representative member of a subgroup of AGS proteins that directly interact with Gβγ. The specific up-regulation of AGS8 in response to hypoxia or ischemia likely represents a component of signal remodeling that occurs during myocyte adaptation to physiological and pathological challenges. Mechanistically, AGS8 appears to promote G protein signaling by a previously unrecognized mechanism for G protein regulation that involves direct interaction with Gβγ.

Results and Discussion

Identification of Receptor-Independent AGSs. Building on the initial strategy that led to the identification of AGS1–3 (12, 13), we undertook a broad screen of cDNA libraries for additional AGS proteins. This screen uses an expression cloning strategy in Saccharomyces cerevisiae to select for receptor-independent activators of the pheromone response pathway in which heterotrimeric G proteins regulate a mitogen-activated protein kinase cascade controlling yeast mating and growth (Fig. 5, which is published as supporting information on the PNAS web site) (12, 13, 19). Our strategy involved the use of a modified yeast strain lacking the pheromone receptor and containing mammalian Go (Goα3, Goα1, or Goαp) in place of the yeast Go subunit. The yeast strain was further modified to respond to activation of the G protein regulated signaling cascade with a readout of growth. This modification...
allowed us to rapidly screen mammalian cDNA libraries constructed in a galactose-inducible vector for activators of this signaling cascade by selecting for those promoting growth in a galactose-specific manner (12, 13, 19). cDNAs isolated with this strategy that required the presence of heterotrimeric G protein for bioactivity were termed AGS proteins and thus are functionally defined rather than based on any conserved protein sequences.

As part of the goal to identify postreceptor signal regulators involved in organ adaptation or disease processes, we screened cDNA libraries from an animal model of cardiac ischemia, human heart, and a prostate leiomyosarcoma (Table 1 and Fig. 5) using modified yeast strains expressing Gαi, Gαo, or Gα16. Sixty-five cDNAs encoding nine distinct proteins were isolated (Gαi strain; 18; Gαo strain; 47; Gα16 strain; 0) with the three cDNA libraries. Using simple growth assays on selective media containing glucose (no cDNA induction) or galactose (cDNA induction) and yeast strains with deletions or modifications of their pheromone signaling pathways we could determine whether the bioactivity of these cDNAs required Gβγ or acted downstream of G protein to influence pathway activation (Figs. 1 and 5) (12, 13, 19). In such epistasis experiments, nine of the cDNAs required Gβγ for their activity and thus were consistent with the definition of a receptor-independent activator of G protein signaling (Table 1). Three of the nine cDNAs encoded the previously characterized AGS1, AGS3, and AGS4 (12, 13, 20). AGS5 and AGS6 encoded truncated versions of the previously characterized proteins LGN and RGS12 respectively and these truncated versions each contained the G protein regulatory (GPR) motif(s) found in these two proteins. The GPR motif inhibits GDP dissociation from Gαi, Gαo, and Gα16 (1, 2, 13, 21, 22). AGS7 encodes the C-terminal region of TRIP13 (CAG33025). AGS9 encodes Rpn10, a component of the 26S proteasome, and AGS10 encodes Gαo, a major Gα protein found in brain. AGS8 represented a truncated version of an uncharacterized mRNA and is described in detail below.

Identification and Bioactivity of AGS8. AGS8 was isolated from the cDNA library generated from a model of repetitive transient ischemia of the heart. In this model, myocardial ischemia was induced by inflation of a pneumatic snare (40 s every 20 min for 8-h periods per day) implanted around the proximal left anterior descending artery (LAD), which leads to marked coronary collateral development in 10 days (E.T. and W.M.C., unpublished observations, and ref. 23). The ischemic area of the left ventricle perfused by the LAD was isolated as “ischemic area” at day 3 and used to generate a cDNA library for screening in the yeast-based expression cloning system described above. This screen yielded six distinct cDNAs: AGS1, AGS3, AGS4, AGS8, or cDNA 1–16, AGS9, and AGS10 (Table 1). AGS8 (cDNA 1–16) encoded the C-terminal 372 aa of an uncharacterized protein KIAA1866 (GenBank accession no. XM.217792.2) plus 621 nt of 3′ untranslated region. Mutation of the first methionine within this terminal 372-aa fragment of the AGS8 cDNA to alanine eliminated its bioactivity in the functional screen, confirming that the reading frame defined within KIAA1866 confers the bioactivity (M.S., W.M.C., and S.M.L., unpublished observations).

As has been reported for AGS1–3 (12, 13, 24), the yeast-based assay system allows rapid determination of the basic mechanisms of action for isolated cDNAs. As mentioned above, epistasis experiments indicated that within the G protein-regulated mitogen-activated protein kinase cascade conferring growth in this yeast strain, AGS8 functioned at the level of G protein itself as its activity was not observed in yeast strains lacking Gβ, Ste20 (p21 activated kinase) or the scaffolding protein Ste5 (Fig. 1A). AGS8 (cDNA 1–16) was active in yeast strains expressing Gαi, Gαo, Gα16, and Gpa1 suggesting that its interaction with G protein may involve Gβγ, which is common to each of the yeast strains (Fig. 1B). AGS8 (cDNA 1–16) was also active in yeast strains expressing a mutant of Gα12 (Gα12ΔG204A), which is postulated to be “locked” in a GDP bound state (12, 13, 25), suggesting that AGS8 is influencing subunit interactions independent of nucleotide exchange (Fig. 1C) (1, 2).

KIAA1866 is a predicted protein from the rat genome containing 4 fibronectin type 3 protein domains, which are found in a large number of intracellular proteins and cell surface receptors (cd00063, www.ncbi.nlm.nih.gov/Structure/cdd/scderv.cgi?uid=cd00063; SM00060, www.ncbi.nlm.nih.gov/Structure/cdd/scderv.cgi?uid=smart00060; pfam00041, www.ncbi.nlm.nih.gov/Structure/cdd/scderv.cgi?uid=pfam00041) (Fig. 24). Full-length AGS8 (GenBank accession no. DQ526268), encoding 1,730 aa, was cloned from a rat heart cDNA library by RT-PCR. The rat cDNA exhibits 67% amino acid identity with the human predicted protein NP_115921 and 86% amino acid

**Table 1. AGS cDNAs isolated in functional screens for receptor-independent AGS**

<table>
<thead>
<tr>
<th>AGS</th>
<th>Protein or gene names in databases and literature</th>
<th>Repetitive transient cardiac ischemia (rat)</th>
<th>Heart (human)</th>
<th>Prostate Leiomysarcoma (human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS1</td>
<td>DexRas1, RASD1</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AGS3</td>
<td>GPSM1 (C-terminal segment with 3–4 GPR motifs)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGS4</td>
<td>G18.1b, GPSM3 (three GPR motifs)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGS5</td>
<td>LGN, GPSM2 (C-terminal 322 amino acids, four GPR motifs)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AGS6</td>
<td>RGS12 (C-terminal 365 amino acids, one GPR motif)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>AGS7</td>
<td>TRIP13</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>AGS8</td>
<td>KIAA1866 (C-terminal 372 amino acids)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AGS9</td>
<td>Rpn10</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>AGS10</td>
<td>Gαo</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

AGS refers to cDNAs isolated in the functional screen and they are numbered according to the order in which they were isolated. Unless indicated otherwise, isolated cDNAs encoded the entire coding sequence. GPR, G protein regulatory motif (13). The rat heart cDNA library contained 2.4 x 10^7 with an average insert size of 2.0 kb. The human heart cDNA library contained 1.5 x 10^7 with an average insert size of 1.3 kb. Number of transformants screened for each library: Repetitive transient ischemic heart (rat), 3.1 x 10^6; prostate leiomyosarcoma (human), 4.3 x 10^6; normal heart (human), 1.0 x 10^6.

dcDNA libraries were screened in yeast strains CY1141 (Gαii), CY8342 (Gαo), and CY9603 (Gα16) (12, 13).
identity with the mouse predicted protein XP.354975 (Fig. 2A). RT-PCR analysis indicated that AGS8 mRNA was expressed in several tissues (Fig. 6, which is published as supporting information on the PNAS web site).

**Regulation of Expression of AGS8 by Ischemia/Hypoxia.** Although AGS8 cDNA was isolated in the yeast screen by using a cDNA library prepared from ischemic myocardium, it was unclear whether it was constitutively expressed or whether it was actually induced in response to the ischemic challenge. Thus, we asked whether AGS8 is induced in the ischemic myocardium. AGS8 mRNA expression was up-regulated 3.5 times in the ischemic area of the left ventricle compared to the nonischemic area in the repetitive transient ischemia model (Fig. 2B). This difference was not observed in the corresponding regions of the heart in nontreated control rats (data not shown). We next asked whether this induction was specific for the ischemic challenge or whether it represented a more general response to myocardial insult. Interestingly, the induction of AGS8 was specific for the transient ischemia model because it was not observed in other cardiac dysfunction models, including sustained tachycardia, hypertrophy, and heart failure (Fig. 2B).

Of the AGS cDNAs isolated in the screen of the repetitive transient ischemia animal model library (AGS1, AGS3, AGS4, AGS8, AGS9, AGS10), only AGS8 mRNA was up-regulated in the ischemic myocardium (M.S., W.M.C., and S.M.L. unpublished observations). We next asked whether the AGS8 mRNA induction observed in the ischemic myocardium reflected changes within the myocyte itself. The hypoxic induction (0% O2 for 1 h) of AGS8 was evaluated in cultured adult left ventricle myocytes, cardiac fibroblasts, rat aortic smooth muscle cells, and rat aortic endothelial cells. The hypoxic induction of AGS8 mRNA was only observed in cultured adult left ventricle myocytes (Fig. 2C). Thus, the induction...
of AGS8 mRNA is specific for the myocyte and the type of stress placed upon the heart.

**AGS8 and G Protein Regulation.** The cardiac- and ischemic-specific induction of AGS8 and its ability to influence G protein signaling in the functional screen suggests that AGS8 may influence G protein signaling mechanisms as part of the myocyte adaptation to hypoxia. To provide a foundation to address this hypothesis, we asked basic questions regarding the interaction of AGS8 with G proteins. The interaction of the bioactive AGS8 cDNA (cDNA 1–16; amino acids A1359-W1730; termed AGS8-C) with G proteins was examined in GST pull-down assays using purified G protein subunits and rat brain lysates. The “Input” lanes contain one-tenth of the mixture used in individual interaction assays. The data are representative of four (A) and three (B) independent experiments.

**Perspective.** Adaptation of the myocardium to ischemia involves alteration of several signaling pathways (i.e., ERK1/2, AKT, protein kinase C, and/or phospholipase C) involved in collateral growth (32, 33), apoptosis (33, 34), energy metabolism (33, 35), and the development of a “preconditioned” myocardium. Gβγ signaling is clearly involved in many aspects of such signaling pathways (36–39). AGS8 provides an unexpected mechanism for regulation of the G protein activation cycle and is specifically induced in the heart in response to transient repetitive ischemia and/or hypoxia. Thus, the receptor-independent regulation of the key signaling cassettes involving Gα and Gβγ may be a very powerful component of organ adaptation and adjustment of the general signaling set point characteristic of individual cells.

**Experimental Procedures**

**Animal Models. Repetitive transient myocardial ischemia.** Repetitive transient myocardial ischemia was achieved in male Wistar rats (~315g) as described (23). Briefly, a pneumatic snare was implanted around the proximal left anterior descending artery (LAD) under isoflurane anesthesia. After 3–4 days of recovery, repetitive transient ischemia was induced by coronary occlusion

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1. Immunoblotting of nondetergent lysates from AGS8-transfected COS cells with AGS8-specific antibody indicated marked enrichment of AGS8 in the pellet fraction obtained by centrifugation at 100,000 × g. AGS8-C and full-length AGS8 generally elicit a small but consistent increase in inositol phosphate production, suggesting that AGS8 is interacting with endogenous G proteins to increase Gβγ-mediated signaling. However, the magnitude of this effect depends on the levels of AGS8 expressed, which is sensitive to the number of plasmids used in the cotransfection experiments.
Increased in the isoproterenol-infused group (beats SEM, ISO 506.7
II-infusion (ATII) (0.75
nitrogen and stored at
were rapidly excised. Left ventricles were rapidly frozen in liquid
After 12 days of infusion, the rats were anesthetized, and hearts
(Model 2002, Altezt, Cupertino, CA) was implanted s.c. Systolic
were obtained from animals killed 21 weeks after surgery in the
rat was created as described (40). Left ventricular tissue samples
quickly separated into the "ischemic area" perfused by the LAD
Figure 4. Influence of AGS8 on Gγ sensitiv signaling pathway. (A) (Left) Influence of AGS8-C on the generation of inositol phosphates. COS-7 cells were
transfected in 12-well plates with control vectors or cDNAs as indicated (0.3 μg of pcDNA3::PLC-β2, 0.3 μg of pcDNA3::Gβγ, 0.2 μg of pcDNA3::Gαs, plus 0.2 μg of pcDNA3::Gαi3, 1 μg of pcDNA3::AGS8-C). The amount of DNA transfected was adjusted to 2 μg per well with pcDNA3 vector. Data are expressed as the percent of inositol phosphates in cells transfected with vector alone (1,002.5 ± 138.5 cpm). (Right) Effect of AGS8 in yeast lacking Go. Yeast strain CY1316 containing empty vector (pRS424) or pRS424::Gαs(E10K) were transformed with empty vector (pYE52) or pYE52::AGS8-C. Data are presented as described in Fig. 1, and are representative of three independent experiments. (B) Influence of full-length AGS8 on the generation of inositol phosphates. COS-7 cells were transfected in 12-well plates with control vectors or cDNAs as indicated (0.3 μg of pcDNA3::PLC-β2, 0.2 μg of pcDNA3::Gβγ, plus 0.2 μg of pcDNA3::Gαs, 1.3 μg of pcDNA3::AGS8). The amount of DNA transfected was adjusted to 2 μg per well with pcDNA3 vector. Data are expressed as the percent of inositol phosphates in cells transfected with vector alone (1,055 ± 163.2 cpm). Data are presented as the mean ± SEM of five (A Left) or eight (B) experiments with duplicate or triplicate determinations. In both A and B, expression of transfected cDNAs was determined by immunoblotting of 10 μg of whole cell lysates. * P < 0.05.

Cardiac hypertrophy and cardiac tachycardia. Male 8-week-old Sprague–Dawley rats (190–210 g) were separated into three groups: Control, saline-infused (CNT); tachycardia, isoproterenol-infusion (ISO) (3
body weight (LV BW) (103):
In the ischemia model of rat heart was used to synthesize cDNAs using SuperScript II (Invitrogen), which were directionally cloned into pDONR222 (Invitrogen) and swapped into the pYES-DEST52 (Invitrogen) and ProQuest normalized human heart cDNA library in pCMVSPORT6 (Invitrogen) and ProQuest normalized human heart cDNA library in pEXP-ADS02 (Invitrogen) were swapped into pYES-DEST52 (Invitrogen) by recombination. Functional screens and growth assays in modified strains of S. cerevisiae were conducted as described (12, 13, 19).

Miscellaneous Procedures. Additional information on materials, AGS8 cloning, AGS8 mRNA analysis, generation of AGS8 antibody, protein interaction assays, cell culture, and data analysis are provided in Supporting Text, which is published as supporting information on the PNAS web site.

This work was supported by National Institutes of Health Grants NS24821 and MH55391 (to S.M.L.). Cell lines were propagated in the Cell and Molecular Imaging Core Facility in the Department of Pharmacology and Center of Excellence in Cardiovascular Research at Louisiana State University Health Sciences Center, supported by the National Institutes of Health Grant P20 RR018766 (S.M.L., program director). S.M.L. is greatly appreciative for support provided by the David R. Bithune/Lederle Laboratories Professorship in Pharmacology and the Research Scholar Award from Yamanouchi Pharmaceutical Company (now named Astellas Pharma).


PNAS | January 17, 2006 | vol. 103 | no. 3 | 801