Activation of Heterotrimeric G-protein Signaling by a Ras-related Protein

IMPLICATIONS FOR SIGNAL INTEGRATION*

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Mary J. Cisowski‡, Chienling Mat‡, Catalina Ribas§, Xiaobing Xie†, Michael Spruyl†, Jeffrey S. Lizio‡, Stephen M. Lanier†, and Emir Duzic‡,**
From JOSI Pharmaceuticals, Tarrytown, New York 10591, the Department of Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425, and Cadus Pharmaceutical Corporation, Tarrytown, New York 10591

Utilizing a functional screen in the yeast Saccharomyces cerevisiae we identified mammalian proteins that activate heterotrimeric G-protein signaling pathways in a receptor-independent fashion. One of the identified activators, termed AGS1 (for getivator of G-protein signaling), is a human Ras-related G-protein that defines a distinct subgroup of the Ras superfamily. Expression of AGS1 in yeast and in mammalian cells results in specific activation of Gαi/Gαo heterotrimeric signaling pathways. In addition, the in vivo and in vitro properties of AGS1 are consistent with it functioning as a direct guanine nucleotide exchange factor for Gαi/Gαo. AGS1 thus presents a unique mechanism for signal integration via heterotrimeric G-protein signaling pathways.

G Protein-Coupled Receptor (GPCR) signaling pathways represent one of the most widely used mechanisms in nature for transmitting signals from the extracellular to the intracellular environment. Each step in the activated GPCR signaling cascade presents a potential regulatory checkpoint for fine-tuning and directing the signal. Although a number of regulatory molecules affecting GPCR signaling have been identified (1–8), evidence suggests the presence of additional post-translational mechanisms (11, 12). As expression of these proteins did not alter G-protein expression levels in yeast, we termed these AGS for getivators of G-protein signaling. This report describes the functional characterization of AGS1, a Ras-related protein isolated from a screen of human liver cDNA.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Plasmid constructions, except as indicated below, have been described previously (11). Plasmid pSVβ-gal was purchased from Promega; pYES2, pCEP4, pcDNA3.1(+), pcDNA3.1-His-locZ, and pcDNA3.1-His-C were from Invitrogen; pYEX4T1 was from Adnami Biotech and pFA2-cjun, pFA2-Erk1, pFA2-CREB, pFA-CHOP, pFB-Luc, pF3-MEK1, and pBluescriptSK(+) were from Stratagene. A plasmid carrying human transducin-α (GNAZ) cDNA sequences in pBluescriptSK(+) was a gift from M. Simon. AGS1 and AGS1-G31V (11) were amplified from pYES2 plasmids and ligated into pcDNA3.1His-C and pYEX4T1, placing the AGS1 coding sequences in frame with, respectively, an N-terminal His₆ tag sequence and an N-terminal GST sequence. In a similar fashion, yeast CDC42 coding sequences were amplified from yeast genomic DNA and ligated into pYEX4T1. The human nociceptor (ORL1) receptor was amplified from brain poly(A)-RNA by reverse-transcriptase polymerase chain reaction and ligated to pCEP4. Transducin-α was excised from pBluescriptSK(+) and ligated to pcDNA3.1(+). Automated dyeoxysequencing was used to verify the correct construction of all plasmids.

Mammalian Transfection and PathDetect Assays— COS-7 cells (ATCC 1651) were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% enhanced calf serum (Gemini Bio-Products), 50 µg/ml penicillin, 50 µg/ml streptomycin, and 100 µg/ml neomycin, pH 7.4. For immunoblot analysis, 1 x 10⁵ cells were transfected with 1 µg of pcDNA3.1-locZ, pcDNA3.1His-AGS1, or pcDNA3.1His-AGS1-G31V and grown 72 h prior to harvesting. Cells were resuspended in 5 ml Tris-HCl, pH 7.4, 0.6 mM EDTA, 5 mM MgCl₂ containing protease inhibitors. PathDetect transfections and luciferase assays were performed in triplicate using 5 x 10⁴ cells/well. Mammalian transfection efficiencies were determined by Western blotting with antibodies recognizing expression from pcDNA3.1His-AGS1, pcDNA3.1His-AGS1-G31V, and pcDNA3.1-locZ. Assays were analyzed by the dual luciferase reporter system (Promega) according to the manufacturer’s instructions.

Mammalian Transduction—Mammalian cell lines were grown in DMEM supplemented with 10% fetal calf serum, pH 7.4. For transient transfections, cells were plated at 0.75 x 10⁵ per well in 12-well plates. Twenty hours after plating, cells were cotransfected with 0.5 µg of pFB-Luc, 0.5 µg of pCF (#5321), and 1 µg of pFB-β-gal. After 24 h, cells were harvested and lysed in a buffer [0.5% Nonidet P-40, 20 mM sodium phosphate, 140 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin, pH 7.4] for 10 min. 


and added to 750 μl of an ice-cold 5% activated charcoal solution in 50 mM NaH₂PO₄. Samples were mixed and centrifuged 5 min at 4 °C. Aliquots of each supernatant (400 μl) were removed and free [³²P]phosphate quantitated by scintillation counting.

In Vivo Labeling of AGS1—Phase II studies of yeast strain CY44 transformed with either pYEX11-T1, pYEX11-T2, pYEX11-T3, or pYEX11-T4 were grown in phosphate-depleted medium (17) for 30 min at 30°C, and 0.2 mM CuSO₄ was added. Thirty min after adding CuSO₄, 0.5 μCi/ml H₂³²P³ (HCl-free; ICN) was added, and cultures were labeled for 5 h. GST fusion proteins were purified essentially as described (11), except proteins were eluted from glutathione-Sepharose at 6 M Urea in 50 mM Tris, pH 8.5, 150 mM NaCl, 1% SDS, 20 mM EDTA, 20 μM phenylmethylsulfonyl fluoride, 2 mM MgCl₂, 2 mM TNP. Protein purity was judged by SDS-polyacrylamide gel analysis, were spotted onto polyethylenimine cellulose plates (J. T. Baker) along with [α-³²P]GTP and [α-³²P]GDP standards. After allowing samples to dry, plates were washed extensively with distilled water followed by methanol, then air-dried. Nucleotides were resolved in 1 M KH₂PO₄, pH 3.4, and detected using x-ray film and intensifying screens at 80°C. The area of sample application (ori) was covered with a lead shield to impede radioactive signal from labeled phosphoproteins and/or phospholipids. 

Activation Assays—Activation assays using purified, recombinant His₆-GSTA1 alone was incubated in assay buffer with 5 μM GTPγS (1.3 × 10⁶ cpm/pmol) and 50-μl aliquots removed at the indicated times for filtration onto nitrocellulose membranes. Filters were air-dried, and bound counts determined in the presence of scintillation fluid.

RESULTS AND DISCUSSION

In yeast screens designed to identify receptor-independent activators of heterotrimeric G-protein signaling we obtained multiple isolates of a single human liver cDNA. Sequence analysis of this cDNA termed AGS1, revealed it to encode a member of the Ras superfamily (Fig. 1). AGS1 possesses all of the consensus guanine nucleotide binding regions of Ras proteins (18) and shares an overall identity of approximately 35% with each of the major Ras subfamilies. AGS1 also contains internal cationic insert regions (amino acids 123–130 and 195–250) not seen in canonical Ras proteins and amino acid variations at amino acids 33, 80, and 82 similar to ones conferring constitutive activity to RhoE (19). A search of the National Center for Biotechnology Information data base revealed AGS1 to be part of a distinct family of eukaryotic Ras-related proteins possessing both these insert regions and variations (Fig. 1). Putative orthologs with 97% identity to AGS1 have been identified in mouse (GenBank™ accession number AF090926; Ref. 20) and rat (GenBank™ accession number AF239157). Closely related human (GenBank™ accession number AL022334) and rat (GenBank™ accession number AF134408; Ref. 21) homologs, each with approximately 80% identity to AGS1, have also been identified. In addition, the C-terminal region of a putative Drosophila gene product (GenBank™ accession number AE003560) shares 49% identity with AGS1.

AGS1 function in yeast was specific for the Gα subclass of heterotrimers and was attenuated in strains either lacking Gβ or expressing a mutated AGS1 carrying a glycine 31 to valine substitution in PM1 (11), a region important for guanine nucleotide binding and hydrolysis. AGS1 function was also attenuated in yeast strains rendered incapable of heterotrimer activation either by mutation of Gα₂ or by co-expression of RGS4 (11). These initial observations suggested that AGS1 functioned in yeast to facilitate GTP exchange on the engineered heterotrimer and that AGS1 function required guanine nucleotide binding and/or hydrolysis.

The activity of AGS1 in yeast presents a totally unexpected paradigm for signal processing in which a monomeric G-protein provides direct input into a heterotrimeric G-protein signaling pathway. To further define its function we determined how AGS1 integrates into GPCR-regulated signaling in mammalian cells. We used a transient expression system in COS-7 cells and the PathDetect luciferase reporter system to evaluate the effect of AGS1 expression on the basal activity of c-Jun N-terminal kinase (JNK), protein kinase A (PKA), p38, and ERK1/2 signaling pathways (22–25).

Activation by AGS1 was specific for the Gα subclass of heterotrimers and that AGS1 function required guanine nucleotide binding and/or hydrolysis.

This selective activation of the ERK1/2 pathway by AGS1 mirrors the Gα selectivity previously seen in the yeast system (11). Furthermore, as many Gα₉ coupled receptors (including the nociceptin receptor) utilize free Gα₉γ to transduce signals through mitogen-activated protein kinase cascades (22–25), this suggests that AGS1 functions in mammalian cells by enhancing Gα₉γ release from Gα₉. Indeed, Elk1 activation by both AGS1 and the activated nociceptin receptor was blocked by cell pretreatment with pertussis toxin, which ADP-ribosylates Gα₉/Go₁, and effectively uncouples it from receptor (26), as well as co-transfection with transducin-α, which attenuates signaling by sequestering free Gγ (Ref. 22; Fig. 2C). In contrast, direct activation of the ERK1/2 signaling pathway by transfection with a plasmid encoding MEK1 was unaffected by either pertussis toxin pretreatment or co-transfection with transducin-α (data not shown). Thus, AGS1 function in mammalian cells appeared mechanistically indistinguishable from that of an agonist-stimulated receptor.

The in vivo function of AGS1 poses many interesting ques-
FIG. 2. Functional analysis of AGS1 in mammalian cells. A, immunoblot analysis of AGS1 protein expression in transfected COS-7 cells. Supernatant (S) and membrane (M) fractions (200 μg each) from cells expressing pcDNA3.1His-lacZ (lacZ), pcDNA3.1His-AGS1 (AGS1), or pcDNA3.1His-AGS1-G31V (G31V) were analyzed by immunoblotting with antiserum raised against the hexahistidine tag sequence (11). Molecular mass markers (in kilodaltons) are indicated. B, AGS1 specifically activates an ERK1/2 signaling pathway in COS-7 cells. Plasmids pcDNA3.1HisC (solid bars) or pcDNA3.1His-AGS1 (open bars) were transfected into COS-7 cells along with pFR-Luc, pSV-β-gal, and either pFA2-cJun (cJun), pFA2-CHOP (CHOP), pFA2-CREB (CREB), or pFA2-Erk1 (Erk1) and relative luciferase activities determined. Basal luciferase activities (in relative luciferase units) were 36,600 ± 3,800, 8,980,000 ± 1,010,000, 78,100 ± 6,700 and 82,500 ± 8,600 for c-Jun, CHOP, CREB, and Erk1, respectively. C, AGS1 functions in a manner analogous to that of a GPCR. Plasmids pcDNA3.1His-AGS1 (AGS1), pcDNA3.1His-AGS1-G31V (G31V), or pCEP4-hNocR (encoding the human nociceptin receptor; hNocR) were transfected into COS-7 cells with plasmids pFA2-Erk1, pFR-Luc, and pSV-β-gal and luciferase activity relative to vector controls determined (open bars). Cells were pretreated with pertussis toxin (shaded bars), co-transfected with transducin-α (solid bars), or stimulated with nociceptin (+ noc) or vehicle (−) as indicated. Basal luciferase activities (in relative luciferase units) were 85,100 ± 11,200 and 89,900 ± 3,400 for HisC and pCEP4 vectors, respectively, and luciferase activity upon direct activation of Erk1 by transfection with a MEK1-encoded plasmid was 4.6 × 10^6 ± 0.2 × 10^6.

FIG. 3. Biochemical properties of purified AGS1. A, GTP hydrolysis activity. Purified GST (○), GST-AGS1 (□), and GST-Cdc42 (■) were incubated with [γ-32P]GTP and aliquots removed at the indicated times to determine free [32P]PO_4 released. Hydrolysis rates were determined by linear regression analysis. B, in vivo labeling of AGS1 with [32P]Pi. Yeast cultures expressing GST, GST-AGS1, or GST-Cdc42 were grown in the presence of [32P]Pi, and labeled nucleotides bound to purified GST fusion proteins were detected by thin-layer chromatography and autoradiography. The migration of free [γ-32P]GDP and [α-32P]GTP standards is indicated.

conditions. These data indicate that GDP resulting from GTP hydrolysis is not stably bound by purified AGS1 and that re-binding of GTP to nucleotide-free AGS1 is rate-limiting, suggesting that AGS1 may associate in vivo with mammalian regulators of nucleotide exchange and/or nucleotide dissociation. Such regulators may mediate stimulus input to AGS1.

Finally, we asked if AGS1 altered the nucleotide binding properties of heterotrimeric G-proteins in vitro. We first measured the ability of purified GST-AGS1 to enhance binding of GTPγS to purified Goα1 and Goα2. Under conditions optimal for monitoring Goα/Goα activation by a GPCR (8), the addition of AGS1 enhanced GTPγS binding to purified Goα1 and Goα2 (Fig. 4A) as well as to purified brain heterotrimeric G-protein (Fig. 4B). AGS1 did not effectively bind GTPγS under these incubation conditions. After a 45-min incubation, the 3-fold increase in GTPγS binding to both free Goα1 and Goα2 in the presence of AGS1 represented 20–30% of the input Goα. This increase in GTPγS binding was not seen when Goα2 was incubated with another purified Ras-related protein, GST-RhoA (data not shown). These observations are consistent with AGS1 functioning in vitro as an exchange factor for Goα/Goα, and activating both free and heterotrimeric Goα.

To confirm that the increase in GTPγS binding in these assays reflected nucleotide binding to Go proteins and not to AGS1, we used both glutathione and nickel-nitrioltriacetic acid affinity resins to re-isolate GST-AGS1 and His₆-Goα₂ following co-incubation. Although we previously used this approach to detect interaction of GST-AGS1 and His₆-Goα₂ (11), aggressive washing of the affinity matrices effectively dissociated the two proteins, as determined by immunoblot analysis (data not shown). After washing >75% of the bound [35S]GTPγS was associated with the nickel-affinity resin, while <5% was associated with the glutathione affinity resin (Fig. 4C). In addition "preloading" Goα₂ with nonradioactive GTPγS prior to association with AGS1 significantly inhibited the subsequent increase in [35S]GTPγS binding (Fig. 4C). These data, as well as the in vivo data both in yeast and in mammalian cells, support a direct role for AGS1 in enhancing GTPγS binding to Goα/Gaγ.

There are several examples of cross-talk between Ras-related protein and heterotrimeric G-protein signaling pathways (25, 28). However, in every instance identified so far, activated heterotrimeric G-protein subunits either activate small G-proteins or work in concert with activated small G-proteins to...
transduce signals. AGS1 is the first example of a monomeric G-protein that functions upstream of a heterotrimeric G-protein to activate it. By virtue of its ability to enhance GTP binding to purified $\alpha_i$, and by its sensitivity to pertussis toxin treatment in vivo, AGS1 appears to function by a mechanism akin to that of a GPCR. It is possible that the cationic regions in AGS1, like those found in the activation loops of many GPCRs to enhance or prolong signaling or may compete with $\alpha_i$ and His$,\alpha_i$S binding assays were performed in triplicate as described under "Experimental Procedures." with either His$,\alpha_i$S alone (No AGS1) or both His$,\alpha_i$S and GST-AGS1 (+AGS1). Total bound [35S]GTP$^\alpha_i$S ($T$) was determined at 30 min on one set by filter binding (open bars). Remaining samples were bound to either nickel-nitrilotriacetic acid (Ni$\text{Ni}$) or glutathione-Sepharose (Gt$\text{Gt}$) column matrices for 30 min, washed three times with 1 ml of assay buffer, and bound counts determined.

Right, His$,\alpha_i$S-GPS (580 nM) was incubated with assay buffer alone (–) or with 20 μM GTP$^\alpha_i$S at 30° for 90 min, dialed twice for 30 min each against 1 liter of ice-cold 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl$_2$, 0.01% Thesit™, 1 mM DTT, then added to purified GST-AGS1 in assay buffer with 5 μM GDP. [35S]GTP$^\alpha_i$S binding assays were performed as described under "Experimental Procedures."

Within the cell AGS1 may work together with activated GPCRs to enhance or prolong signaling or may compete with GPCRs for activation of heterotrimeric G-proteins. Alternatively AGS1 may function in an independent signaling pathway, activating GPCR- or non-GPCR-coupled heterotrimeric G-proteins either intracellularly or at the cell surface. The unusual nucleotide binding properties of purified AGS1, as well as its abundant transcription in a variety of tissues (not shown), suggests the existence of regulators of AGS1 function. The identity of these putative regulators remains to be determined.

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REFERENCES


FIG. 4. AGS1 facilitates nucleotide exchange on heterotrimeric G$\alpha_i$. A, activation of recombinant G$\alpha_i$ and His$\alpha_i$G$\alpha_i$ by purified AGS1. G$\alpha_i$ proteins were incubated either alone ( ), with GST ( ), or with GST-AGS1 ( ) prior to the addition of [35S]GTP$^\alpha_i$S. A control sample with AGS1 alone at 30 min ( ) is indicated. Bound nucleotide was determined at the indicated times after addition of label. B, activation of brain heterotrimeric G-protein by AGS1. Purified brain heterotrimer, GST, and GST-AGS1 (AGS1) were incubated either alone or in combination prior to purified GST-AGS1 in assay buffer with 5 mM GDP. [35S]GTP$^\alpha_i$S binding assays were performed in triplicate as described under "Experimental Procedures."
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