Identification of a Truncated Form of the G-protein Regulator AGS3 in Heart That Lacks the Tetratricopeptide Repeat Domains

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AGS3, a 650-amino acid protein encoded by an ~4-kilobase (kb) mRNA enriched in rat brain, is a Goi/Goqi binding protein that competes with Gβγ for interaction with GoiGDP and acts as a guanine nucleotide dissociation inhibitor for heterotrimeric G-proteins. An ~2-kb AGS3 mRNA (AGS3-SHORT) is enriched in rat and human heart. We characterized the heart-enriched mRNA, identified the encoded protein, and determined its ability to interact with and regulate the guanine nucleotide-binding properties of G-proteins. Screening of a rat heart cDNA library, 5'-rapid amplification of cDNA ends, and RNase protection assays identified two populations of cDNAs (1979 and 2134 nucleotides plus the polyadenylation site) that diverged from the larger 4-kb mRNA (AGS3-LONG) in the middle of the protein coding region. Transfection of COS-7 cells with AGS3-SHORT cDNAs resulted in the expression of a major immunoreactive AGS3 polypeptide (Mr ~23,000) with a translational start site at Met465 of AGS3-LONG. Immunoblots indicated the expression of the Mr ~23,000 polypeptide in rat heart. Glutathione S-transferase-AGS3-SHORT selectively interacted with the GDP-bound versus guanosine 5'-O-(3-thiotriphosphate) (GTPγS)-bound conformation of Goi2 and inhibited GTPγS binding to Goi2. Protein interaction assays with glutathione S-transferase-AGS3-SHORT and heart lysates indicated interaction of AGS3-SHORT with Go112 and Go122 but not Goi or Go1i2. Immunofluorescent imaging and subcellular fractionation following transient expression of AGS3-SHORT and AGS3-LONG in COS-7 and Chinese hamster ovary cells indicated distinct subcellular distributions of the two forms of AGS3. Thus, AGS3 exists as a short and long form, both of which apparently stabilize the GDP-bound conformation of Goi, but which differ in their tissue distribution and trafficking within the cell.

G-proteins serve as primary transducers for propagation of signals initiated by the superfamily of G-protein-coupled recep-

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1 The abbreviations used are: kb, kilobase(s); TPR, tetratricopeptide repeat; GPR, G-protein regulatory; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; nt, nucleotide(s); UTR, untranslated region; CHO, Chinese hamster ovary; CWS, cell washing solution; GTPγS, guanosine 5'-O-(3-thiotriphosphate).

2 The GPR motif was also termed the GoLoco motif (11).

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long forms that differ in their tissue distribution and trafficking within the cell.

**EXPERIMENTAL PROCEDURES**

**Materials—**[32P]UTP (800 Ci/mmol) was purchased from PerkinElmer Life Sciences. Tissue culture supplies were obtained from JRH Biosciences (Lenexa, KS). Acrylamide, bisacrylamide, protein assay kits, and SDS were purchased from Bio-Rad. Guanosine diphosphate was obtained from Roche Molecular Biochemicals. Polyvinylidene difluoride membranes were obtained from Pall Gelman Sciences (Ann Arbor, MI). Polyolonal Goαi antisera generated against the carboxyterminal 10 amino acids was kindly provided by Dr. Thomas W. Gettys (Department of Medicine, Medical University of South Carolina) (12). Superscript II, LipofectAMINE, and the 5′-RACE kit were obtained from Life Technologies, Inc. Marathon-Ready cDNA and multiple-tissue blots for rat and human mRNAs were obtained from CLONTECH. Goαi, expressed in and purified from Sf9 cells, was kindly provided by Stephen G. Graber (Department of Pharmacology, West Virginia University School of Medicine) (13). All other materials were obtained as previously described (6, 14, 15).

**RNA Blot Analysis—**Rat brain and heart RNAs were isolated from 220-g Harlan Sprague-Dawley rats by extraction with RNAzol (TEL- TES, TX). Total RNA (50–70 μg) was electrophoresed on 3% formaldehyde-containing 1% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) under vacuum. The membrane was baked for 2 h at 80 °C in a vacuum oven and prehybridized and hybridized in phosphate buffer containing 0.5 M NaH2PO4, pH 7.2, 1% bovine serum albumin, 75% SDS, and 1 mM EDTA as previously described (16). 32P-Labeled cDNA probes were generated by random priming using the MultiPrime kit (Amersham Pharmacia Biotech). Blots were hybridized with the probe for 12 h at 65 °C, and the membrane was washed twice (40 mM Na2HPO4, pH 7.2, 5% SDS, and 1 mM EDTA) at 65 °C and exposed to XAR-5 film for 2–4 days at −70 °C.

**Library Screening—**A rat heart cDNA library (Uni-ZAP XR, lot 937512, Stratagene) was screened with a [32P]labeled 40-mer oligonucleotide corresponding to the open reading frame of the cDNA (kit from Life Technologies, Inc.) and Marathon-Ready cDNA, respectively, the amplified products were eluted from the gel and subcloned into the TA cloning vector pCR-TOPO (Invitrogen, San Diego, CA). Bacterial colonies obtained from these transformants were again screened by hybridization with the same AGS3 oligonucleotide. Plasmids from positive colonies were purified, and the nucleotide sequence of the insert was determined in the DNA Sequencing Facility at the Medical University of South Carolina.

**RNase Protection Assays—**Total and cellular mRNAs were isolated as described above. Riboprobes were labeled with [32P]UTP (800 Ci/ mmol), and RNase protection assays were performed using the Ambion series of pre-made reagents as described (15). RNA probes were generated from fragments of AGS3-SHORT cDNAs that spanned the region of sequence divergence from AGS3-LONG, thus allowing the detection of both AGS3-LONG and AGS3-SHORT transcripts in the same sample. To generate constructs for probe generation, an AGS3-SHORT-1 cDNA obtained by 5′-RACE was isolated from pCR-TOPO by restriction with Nael and EcoRI and subcloned into the EcoRI and Smal sites of pBluescript SK. For AGS3- SHORT-2, the longest cDNA isolated by library screening was restricted with EcoRI and Smal and subcloned into the EcoRI and Smal sites of pSK.Bluexpress. The vectors were linearized by Xhol, and 32P-labeled antisense riboprobes were made using T3 RNA polymerase. 1 μg of heart or brain mRNA or 0.5 μg of CHO total RNA was hybridized with probe for 12 h at 45 °C, followed by RNase A/RNase T1 digestion. Protected fragments were separated on an 8 m urea and 5% polyacryl- amide gel and visualized by exposure to X-Omat film at −90 °C for 12–24 h. The respective insert and probe lengths for AGS3-SHORT-1 was 447 nt. The respective insert sizes for AGS3-SHORT-2 were 230 and 319 nt. The sizes of the protected fragments resulting from annealing the AGS3-SHORT-1 and AGS3- SHORT-2 probes with AGS3-LONG would be 228 and 184 nt, respectively.

**Cell Transfection and Tissue Fractionation—**CHO and COS-7 cells were grown in Ham's F-12 medium and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml). For transient expression, CHO and COS-7 cells at 70–80% confluency (100-mm dish) were transfected with 10 μg of AGS3-LONG or AGS3-SHORT cDNA using 30 μl of LipofectAMINE. Cells were harvested after 48 h and resuspended in lysis buffer (5 mM Tris- HCl, 5 mM EDTA, 5 mM EGTA, pH 7.4, 1 mM phenylmethylsulfonyl, 0.5 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotinin) with a 25G gauge syringe. The samples were centrifuged at 100,000 × g for 30 min at 4 °C to generate a crude membrane pellet and a 100,000 × g supenatant that represents the cytosol. The crude membrane pellet was washed once with 4 volumes of membrane buffer. Rat heart or brain tissue was homogenized in lysis buffer using a Powergen 125 tissue disruptor (Bio-merieux, Montalbe, France). Aliquots of samples were added to Laemmli sample buffer and placed in a boiling water bath for 5 min prior to processing by SDS-polyacrylamide gel electrophoresis and immunoblotting as previously described (6). Three AGS3 peptides (PEP22 (Asg255–Gly300), PEP98 (Val425–Ser600), and PEP22 (Thr607–Ile859)) from different areas of AGS3-LONG were synthesized and conjugated for generation of rabbit polyclonal antisera in the Peptide Synthesis and Antibody Production Facility at the Medical University of South Carolina. Each of these peptides was affinity purified prior to use.

**Immunofluorescence Microscopy—**CHO and COS-7 cells were plated onto coverslips (18-mm round no. 1) precoated with 0.1% polylysine and transfected with AGS3-SHORT-1, AGS3-LONG, or AGS3-TPR in peDNA. The AGS3-TPR construct consisted of the first 454 amino acids of AGS3-LONG. AGS3-TPR was generated by PCR using primers 5′-GGAGTTACGTACGATCATGGGAGGACT-3′ (forward) and 5′-GGGTGAGACGATGCTGCAG-3′ (reverse), in which amino acid codon 455 of AGS3-LONG was mutated to stop codon. 48 h after transfection, cells were briefly washed with cell washing solution (CWS, 137 mM NaCl, 2.6 mM KCl, 1.8 mM KH2PO4, and 10 mM Na2HPO4) and fix ed for 5 min in CWS containing 3% paraformaldehyde. Fixed cells were rinsed with CWS (3 × 5 ml/coverslip) and incubated with 0.05% Triton X-100 for 1 min at room temperature and washed twice with CWS (3 ml/coverslip). The coverslips were then incubated with CWS containing 1% fetal bovine serum for 30 min at 24 °C, followed by incubation with a 1:100 dilution of affinity-purified AGS3 PEP22, PEP32, or PEP98 antibody in CWS for 14 h at 4 °C. Coverslips were washed three times with CWS (3 ml/coverslip) and incubated with secondary antibody (fluorescein isothiocyanate).
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Tissue Distribution of AGS3 mRNA—AGS3 was initially defined as a 650-amino acid protein encoded by an ~4-kb cDNA (6). To determine the tissue distribution of the AGS3 mRNA, we performed mRNA blot analysis with two probes derived from the upstream and downstream regions of the ~4-kb mRNA coding region (Fig. 1A). Both probes identified an ~4-kb mRNA enriched in brain (Fig. 1B). The ~2-kb mRNA was also identified with hybridization probes derived from the 3′-UTR of the ~4-kb cDNA. The relative expression of the ~4- and ~2-kb AGS3 mRNAs in heart was developmentally regulated. In rat heart, the ~4-kb mRNA was enriched at birth, whereas the ~2-kb mRNA predominated in the adult rat (Fig. 1C). Within the adult rat heart, the ~2-kb mRNA was clearly enriched in ventricular tissue. Although a full-length human AGS3 cDNA is not available, we obtained a partial human AGS3 expressed sequence tag clone and used this sequence to perform a similar RNA blot analysis in human tissue (Fig. 2). The human AGS3 probe (344 nt) corresponded to the same region of AGS3 used to generate PROBE B in Fig. 1 and thus would detect both AGS3-SHORT and AGS3-LONG. In human, as in rat, there were two AGS3 transcripts of ~2 and 4 kb that were differentially enriched in heart and brain (Fig. 2).

3 N. Pizzinat and S. M. Lanier, unpublished observations.
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As in rat heart, the ∼4-kb AGS3 mRNA in human heart predominated at birth, whereas both mRNAs were expressed in the adult heart (Fig. 2). The ∼4-kb mRNA and its encoded protein were termed AGS3-LONG, whereas the ∼2-kb mRNA was termed AGS3-SHORT, and we initiated experiments to define the smaller mRNA and its encoded protein.

Identification of AGS3-SHORT cDNAs—To define the AGS3-SHORT transcript, we screened a rat heart cDNA library, identified the 5′ terminus by RACE, and performed RNase protection experiments. A rat heart cDNA library was screened with an oligonucleotide derived from the 3′-UTR of AGS3-LONG. 11 individual cDNAs were isolated and sequenced. The sizes of the cDNAs ranged from 1509 to 2134 nt, and each contained a 3′-UTR (∼1.3 kb) identical to that of AGS3-LONG. Three identical cDNAs (AGS3-SHORT-1) contained 114 nt of 5′-terminal sequence that diverged from that of AGS3-LONG in the linker domain of the protein coding region of AGS3-LONG (Fig. 3). A fourth cDNA (AGS3-SHORT-2) contained 46 nt of 5′-terminal sequence that also diverged from that of AGS3-LONG in the linker region, and this 5′-sequence was distinct from that of AGS3-SHORT-1. The lengths of AGS3-SHORT-1 and AGS3-SHORT-2 cDNAs (1979 and 2134 nt plus the poly(A) tail) were similar to the size of the AGS3-SHORT mRNA identified by RNA blot analysis. The remaining cDNAs isolated from the rat heart library also terminated in the linker region, but did not diverge from the AGS3-LONG sequence, likely representing incomplete processing of the mRNA during reverse transcription.

To further define the population of AGS3-SHORT mRNAs in heart, we performed 5′-RACE by different methods with two different sources of rat heart mRNA. AGS3-SHORT-1 and AGS3-SHORT-2 were identified by 5′-RACE in both experimental methods. In the first method, mRNA was isolated from rat ventricles and reverse-transcribed with an AGS3-specific primer corresponding to sequence in the 3′-UTR of AGS3-LONG, and the AGS3-SHORT cDNAs were identified by library screening. The generated cDNAs were dC-tailed at the 5′-end and amplified with an anchor primer and a series of nested primers downstream from the 5′-terminus of the AGS3-SHORT cDNAs identified by library screening. The second method involved a similar amplification strategy with different AGS3 nested primers, but used a commercial source of double-stranded cDNAs from rat heart as template. The two methods were selected to take advantage of the different 5′-tailing procedures and to evaluate different sources of mRNA. AGS3-SHORT-1 was represented by 11 of 14 cDNAs in the first method and by 10 of 18 cDNAs in the second method. The longest AGS3-SHORT-1 cDNAs identified by 5′-RACE contained 144 nt of 5′-terminal sequence that diverged from that of AGS3-LONG (Fig. 3). AGS3-SHORT-2 was identified (3 of 18) only in the second method. The remaining cDNAs (3 of 14 in the first method and 5 of 18 in the second method) contained various lengths of sequence that did not diverge from AGS3-LONG and likely represented incomplete extensions of the larger cDNA. Thus, AGS3-SHORT-1 and AGS3-SHORT-2 cDNAs were identified in three procedures using different sources of RNA and experimental strategies. In each case, AGS3-SHORT-1 cDNA was the predominant species.

Finally, we confirmed the expression of AGS3-SHORT cDNAs in heart by RNase protection assays using a probe design that allowed us to identify the AGS3-LONG and AGS3-SHORT cDNAs in the same reaction (Fig. 3A). To provide internal controls for observed signals, CHO cells were transiently transfected with AGS3-SHORT or AGS3-LONG cDNAs. As illustrated in Fig. 3B, RNase protection assays with the AGS3-SHORT-1 cDNA probe yielded two protected fragments in heart of ∼350 and ∼230 nt, corresponding to the predicted sizes of AGS3-SHORT-1 and AGS3-LONG (Fig. 4B), respectively. Due to the design of the AGS3-SHORT-1 probe, the signal in heart and brain actually represents the sum total of both AGS3-LONG and AGS3-SHORT-2 mRNAs. RNase protection assays with the AGS3-SHORT-2 cDNA probe (Fig. 4C) also yielded two protected fragments in heart and brain of ∼230 and ∼190 nt, corresponding to the predicted sizes of AGS3-SHORT-2 and AGS3-LONG, respectively. The two protected species corresponding to AGS3-SHORT and AGS3-LONG were the two major fragments detected in the assay, indicating the absence of any other heterogeneity in this region of the transcript. The two major protected species were also observed with both probes using brain RNA, although the AGS3-LONG fragment clearly predominated in brain tissue. Based upon the relative intensities of the AGS3-SHORT and AGS3-LONG protected species in Fig. 4 (B and C), it is also clear that the AGS3-SHORT-1 cDNA is the major AGS3-SHORT transcript, as suggested by the results obtained with library screening and 5′-RACE.

These data indicate that the AGS3-SHORT and AGS3-LONG transcripts contain identical 3′-UTRs and share a segment of the AGS3-LONG coding region, but differ in their 5′ termini. The 5′-end of AGS3-SHORT mRNAs terminates in the linker domain of the protein coding region of AGS3-LONG and as such may encode a “truncated” version of the protein encoded by AGS3-LONG. This truncated version would be of particular interest as it would contain the GPR motifs that regulate Gαi, but would lack the tetratricopeptide repeats found in the amino-terminal half of the protein.

Identification of the Protein Encoded by AGS3-SHORT cDNAs—Analysis of the open reading frames for AGS3-SHORT cDNAs indicated that the longest open reading frame corresponded to the carboxyl-terminal third of the protein encoded by AGS3-LONG (Fig. 5) and that the divergent 5′-sequence of the AGS3-SHORT cDNAs was not in frame with the AGS3 protein coding sequence. For AGS3-SHORT-1, the major transcript in heart, the longest open reading frame began at nt 217, encoding a protein of 166 amino acids with a calculated Mr of 18,117. AGS3-SHORT-2 contains a slightly longer portion of AGS3-LONG at its 5′-end, and the longest open reading frame would begin at nt 189, encoding a protein of 227 amino acids with a calculated Mr of 25,076. The longest open reading frames for the AGS3-SHORT transcripts would initiate at me-
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To define the encoded protein, we transiently expressed AGS3-SHORT cDNAs in COS-7 and CHO cells and performed immunoblot analysis with antisera generated against peptides derived from different regions of AGS3-LONG (Fig. 6A). PEP22 and PEP98 antisera would recognize AGS3-LONG and any AGS3-related proteins encoded by the AGS3-SHORT transcripts. PEP32 would recognize AGS3-LONG, but would not recognize any AGS3-related proteins encoded by the AGS3-SHORT-1 transcripts. The first in-frame methionine for AGS3-SHORT-2 is in the middle of the peptide sequence used to generate PEP32 and thus may or may not be recognized by PEP32 depending upon the epitope (Fig. 6A). Immunoblot analysis indicated that both AGS3-SHORT cDNAs encoded a major Mr; 23,000 polypeptide (Fig. 6B). The Mr; 23,000 peptide was not observed in immunoblots with PEP32 (Fig. 6B). The Mr; 23,000 peptide was not observed following transfection of COS-7 cells with pcDNA3-AGS3-SHORT-1M*, in which the first in-frame methionine was eliminated by site-directed mutagenesis (Fig. 6C). The start methionine for the Mr; 23,000 peptide corresponds to the first in-frame methionine for AGS3-SHORT-1 (nt 217) and the second in-frame methionine for the AGS3-SHORT-2 cDNAs identified by library screening and 5'-RACE. Two populations of heart AGS3 cDNAs (AGS3-SHORT-1 and AGS3-SHORT-2) were identified as described under “Experimental Procedures.” The sequence unique to AGS3-SHORT cDNAs is indicated by lowercase letters. The arrows in the 3'-UTR and the coding region of AGS3-LONG indicate nested primers used for PCR amplification in 5'-RACE in the first and second experimental methods as described under “Experimental Procedures.” The hatched and solid bars within the AGS3-LONG coding region correspond to TPR and GPR motifs, respectively. The stippled and striped segments of AGS3-SHORT cDNAs indicate sequence divergence from AGS3. The sequences of the unmarked open segment of AGS3-SHORT cDNAs and the 3'-UTR of AGS3-SHORT cDNAs were identical to those of AGS3-LONG.

FIG. 3. AGS3-SHORT cDNAs identified by library screening and 5'-RACE. Two populations of heart AGS3 cDNAs (AGS3-SHORT-1 and AGS3-SHORT-2) were identified as described under “Experimental Procedures.” The sequence unique to AGS3-SHORT cDNAs is indicated by lowercase letters. Upper case letters indicate sequence identity to AGS3-LONG. The arrows in the 3'-UTR and the coding region of AGS3-LONG indicate nested primers used for PCR amplification in 5'-RACE. The first and second experimental methods as described under “Experimental Procedures.” The hatched and solid bars within the AGS3-LONG coding region correspond to TPR and GPR motifs, respectively. The stippled and striped segments of AGS3-SHORT cDNAs indicate sequence divergence from AGS3. The sequences of the unmarked open segment of AGS3-SHORT cDNAs and the 3'-UTR of AGS3-SHORT cDNAs were identical to those of AGS3-LONG.

FIG. 4. RNase protection experiments with probes derived from AGS3-SHORT cDNAs. A, the regions used to generate riboprobes for AGS3-SHORT cDNAs are indicated by the thick lines. The probes thus overlap the point at which the sequence of AGS3-SHORT cDNAs diverged from that of AGS3-LONG as described under “Experimental Procedures.” Refer to the Fig. 3 legend for additional details. B and C, AGS3-SHORT cDNAs were inserted into pcDNAs and transiently expressed in CHO cells as described under “Experimental Procedures” to provide internal controls. 1 μg of mRNA from brain or heart and 0.5 μg of total RNA from CHO transfectants were incubated with ~300,000 cpm AGS3-SHORT-1 (B) or AGS3-SHORT-2 (C) riboprobes at 45 °C for 12 h, and hybrids were treated with RNases, followed by precipitation. The pellets were resuspended in 20 μl of loading buffer and resolved on urea-5% polyacrylamide gels. 3 μl of the CHO transfectant samples and 10 μl of the tissue samples were loaded in B. 5 μl of the CHO transfectant, 5 μl of the brain samples, and 15 μl of the heart samples were loaded in C. In B, the AGS3-SHORT-1 cDNA used for CHO transfection actually lacked 15 nt at the 5'-end found in the AGS3-SHORT-1 RNA used for probe generation and thus resulted in the slightly smaller size observed with CHO versus heart RNA. In C, the AGS3-SHORT-2 cDNA used for CHO transfection and probe generation shared an extra 9 nucleotides from the vector, thus accounting for the slightly larger size observed with CHO versus heart RNA.

thionines within the AGS3-LONG sequence itself. Neither of these methionines is placed in the context of an optimal Kozak consensus sequence for initiation of translation (17).

To define the encoded protein, we transiently expressed AGS3-SHORT cDNAs in COS-7 and CHO cells and performed immunoblot analysis with antisera generated against peptides derived from different regions of AGS3-LONG (Fig. 6A). PEP22 and PEP98 antisera would recognize AGS3-LONG and any AGS3-related proteins encoded by the AGS3-SHORT transcripts. PEP32 would recognize AGS3-LONG, but would not recognize any AGS3-related proteins encoded by the AGS3-SHORT-1 transcripts. The first in-frame methionine for AGS3-SHORT-2 is in the middle of the peptide sequence used to generate PEP32 and thus may or may not be recognized by PEP32 depending upon the epitope (Fig. 6A). Immunoblot analysis indicated that both AGS3-SHORT cDNAs encoded a major Mr; 23,000 polypeptide (Fig. 6B). The Mr; 23,000 peptide was not observed in immunoblots with PEP32 (Fig. 6B). The Mr; 23,000 peptide was not observed following transfection of COS-7 cells with pcDNA3-AGS3-SHORT-1M*, in which the first in-frame methionine was eliminated by site-directed mutagenesis (Fig. 6C). The start methionine for the Mr; 23,000 peptide corresponds to the first in-frame methionine for AGS3-SHORT-1 (nt 217) and the second in-frame methionine for...
Thus, the peptide likely reflects translational initiation at the same methionine for both AGS3-SHORT cDNAs.

The AGS3-SHORT-2 cDNA also encoded two additional immunoreactive species (M_r = 32,000 and 34,000) upon COS-7 or CHO cell transfection. Both the M_r = 32,000 and 34,000 peptides were recognized by AGS3 PEP32, PEP22, and PEP98 antisera (Fig. 6B). The M_r = 32,000 peptide was not observed following transfection of COS-7 cells with pcDNA3-AGS3-SHORT-2M*, in which the first start methionine (nt 189) in frame with the AGS3 coding sequence was eliminated by site-directed mutagenesis (Fig. 6C). The immunoreactive signal for the M_r = 32,000 peptide...
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3.5 GPR motifs. The TPR regions of the Drosophila AGS3 homolog PINS play a key role in the positioning of the protein within the cell (21, 22). Thus, AGS3-SHORT and AGS3-LONG may follow different trafficking patterns within the cell. To more specifically address this issue, we performed immunofluorescent experiments to determine the subcellular location of AGS3-SHORT and AGS3-LONG following transient expression in COS-7 and CHO cells. Immunofluorescent analysis of AGS3 expression indicated clear differences in the subcellular distribution of the long and short AGS3 variants (Fig. 8). AGS3-SHORT was found throughout the cell, with a diffuse distribution in the cytosol. AGS3-LONG exhibited more of a punctate distribution, non-homogeneously distributed in the cell cytoplasm. The subcellular distribution of AGS3-TPR, which contained the first 454 amino acids of AGS3 and thus lacked the GPR domains, was identical to that of full-length AGS3-LONG. The subcellular distributions of AGS3-SHORT, AGS3-LONG, and AGS3-TPR were similar in both COS-7 and CHO cells. Another construct in which the AGS3-SHORT sequence was extended upstream to the carboxyl-terminal end of TPR-VII exhibited a subcellular distribution identical to that of AGS3-SHORT. These data indicate that the TPR domains indeed account for the differences in the targeting of AGS3-SHORT and AGS3-LONG within the cell.

Multiple experiments in our laboratory are consistent with this point, particularly if one evaluates the relative distribution of the two forms of AGS3 in a 100,000 × g pellet and supernatant following cell lysis. Subcellular fractionation indicated that AGS3-SHORT readily partitioned into a 100,000 × g supernatant in rat heart and following transient expression in mammalian cells (Figs. 6 and 7). In contrast, AGS3-LONG was found in both the 100,000 × g pellet and supernatant following transient expression in mammalian cells. The relative distribution of AGS3-LONG in the 100,000 × g pellet versus supernatant was sensitive to the method of cell fractionation and varied in different cell types. Repeated washing of the 100,000 × g pellet releases the majority of AGS3-LONG into the supernatant, as observed for preparations from rat brain and primary cultures of cortical neurons and transfected cells (8). In contrast, essentially all of the AGS3-SHORT was found in the 100,000 × g supernatant independent of pellet washing. These data suggest a poorly understood, loose association of AGS3-LONG with a binding partner that retains AGS3-LONG in a different cellular compartment compared with AGS3-SHORT.

Interaction of AGS3-SHORT with G-proteins Results in Inhibition of Guanine Nucleotide Exchange and Movement of AGS3-SHORT to a Membrane Fraction—To define the functional influence of AGS3-SHORT on G-protein, we first evaluated the interaction of AGS3-SHORT with purified Goα12. AGS3-SHORT was generated as a GST fusion protein and incubated with purified Goα12 in the presence of GDP or GTPγS. G-protein bound to AGS3-SHORT was isolated on a glutathione affinity matrix and identified by immunoblotting (Fig. 9A). AGS3-SHORT selectively interacted with the GDP-bound conformation of Goα12. These studies were then extended to determine the effect of AGS3-SHORT on the nucleotide-binding properties of Goα12. AGS3-SHORT effectively inhibited the binding of GTPγS to Goα12 (Fig. 9B), consistent with the idea that the interaction of AGS3-SHORT with Goα12 stabilizes the GDP-bound conformation of Goα. Further studies with rat heart lysates indicated a preference of AGS3-SHORT for the Gi family of proteins (Fig. 9C). GST-AGS3-SHORT bound Goα12 and Goα3, but not Goa,

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4 M. Bernard and S. M. Lanier, unpublished observations.
5 J. Blumer, S. M. Lanier, and J. Chandler, unpublished observations.
Ga or Gq. Despite the presence of added GDP, which would stabilize the heterotrimeric G-protein complex, Gbg was not associated with the Ga bound to AGS3 (Fig. 9C).

Although AGS3-SHORT and AGS3-LONG both bind Goa, the relative distribution of AGS3 and G-proteins within the cell differs, indicating that only a subpopulation of the two proteins may be complexed at any given time. A key question is what determines the subcellular location of AGS3 and if the trafficking of AGS3 is regulated by signals from inside or outside of the cell. As an initial approach to this issue, we investigated if coexpression of AGS3-SHORT or AGS3-LONG with Goa influenced the amount of AGS3 or Goa found in the 100,000 × g pellet following transient expression in COS-7 or CHO cells. Coexpression of AGS3-SHORT with Goa resulted in the movement of the AGS3-SHORT protein from the 100,000 × g supernatant to the 100,000 × g pellet (Fig. 10). The already substantial portion of AGS3-LONG found in the 100,000 × g pellet was not altered by coexpression of Goa. In cells transfected with pcDNA3-Goa, essentially all of the Goa protein was in the 100,000 × g pellet, and this was not altered by coexpression of Goa.
AGS3-SHORT or AGS3-LONG. The translocation of AGS3-SHORT by $G_{\alpha_i}\mathrm{GTP}
$ was specific, as there was only minimal translocation of AGS3-SHORT following overexpression of $G_{\alpha_i}$ (Fig. 10), which is consistent with the protein interaction experiments in which GST-AGS3-SHORT selectively bound $G_{\alpha_i}$ versus $G_{\alpha_i}$ in heart cell lysates (Fig. 10).

**DISCUSSION**

The response of the cell to external stimuli has become progressively more intricate as higher organisms have evolved. Nature has certainly found the seven-membrane span motif along with its “partner” G-proteins to be one of the most efficient mechanisms for selective processing of spatially and temporally delineated signals by the cell. As the cell found itself challenged by a number of diverse stimuli, it evolved various mechanisms of integration to handle these inputs. The seven-membrane span motif has become more and more diverse in terms of the stimuli that it recognizes, and G-proteins have evolved by subunit diversification, differences in guanine nucleotide-binding/hydrolysis properties, and variations in post-translational modifications (23). Mechanisms for signal integration also include stoichiometric considerations, signal cross-talk, and spatial restriction of signaling components within the cell.

Additional specialized mechanisms for fine-tuning signal processing include receptor regulation and non-receptor proteins/lipids that influence subunit interactions and the activation state of G-proteins. Such entities include a number of proteins identified in protein interaction or functional screens. AGS3 is one of three proteins isolated as a G-protein regulator in a functional screen. This report indicates the expression of two forms of AGS3 (AGS3-SHORT and AGS3-LONG) that are differentially enriched in heart and brain. The two forms of AGS3 essentially differ in the presence and absence of the TPR domains found in the amino-terminal half of AGS3-LONG. The mechanism by which AGS3 is generated as two forms is unclear. Although the AGS3 (rat or human) gene is not defined, the AGS3-related protein in Caenorhabditis elegans is encoded by 14 exons (GenBank™/EBI accession number U40409) (8). Thus, the two forms of AGS3 may be generated by alternative splicing, trans-splicing or even alternative promoters. Data base searches (BLAST) with the divergent 5′-ends of AGS3-SHORT cDNAs indicated no matches with AGS3-SHORT-2; however, the AGS3-SHORT-1-specific sequence was identical to a segment of the 5′-UTR of OMP25 (24). Whereas AGS3-LONG is the major species in adult rat brain, AGS3-SHORT is the major AGS3 protein expressed in adult rat heart. The relative expression of the two forms of AGS3 is developmentally regulated and apparently varies among the different regions of the heart.

At a structural level, the major differences between the short and long form of AGS3 are due to the absence in AGS3-SHORT of the seven TPR domains found in AGS3-LONG. This likely has important implications in terms of regulation of the function of AGS3-SHORT and AGS3-LONG. The TPR domain of the AGS3-LONG-related protein in Drosophila, PINS, is required for translocation of the protein to a specific membrane domain of the neuroblast, and this is achieved by the binding of the protein INSCUTEABLE to the TPR domains of PINS. The TPR motif is a highly degenerate sequence found in a large number of proteins, where it suberves various functions via protein interactions. The absence of the TPR motif in the AGS3-SHORT protein apparently accounts for the differences in subcellular distribution of the two forms of AGS3. The generation of two forms of AGS3 that apparently differ in subcellular trafficking and tissue distribution, but yet both interact with $G_{\alpha_i}$ subunits, is of particular interest. The two forms of AGS3 may play different roles in G-protein-regulated events.

AGS3-LONG in rat brain (8) and the AGS3-LONG-related protein PINS in Drosophila melanogaster (21, 22) are complexed with $G_{\alpha_i}$ and AGS3-SHORT selectively interacts with $G_{\alpha_i}$ proteins in heart. The regions of AGS3 interacting with G-proteins are localized to the GPR motifs in the carboxy-terminal half of the protein (6, 8), which are present in both AGS3-SHORT and AGS3-LONG. Each GPR motif in AGS3 appears capable of binding $G_{\alpha_i}\mathrm{GDP}$ (8). Indeed, protein interaction experiments with a GST-AGS3 fusion protein containing four GPR motifs indicate that multiple $G_{\alpha_i}$ subunits may be tethered to AGS3, suggesting a role for the protein within a larger signal transduction complex (8). The interaction of AGS3 GPRs with $G_{\alpha_i}$ or $G_{\alpha_i}$ actually inhibits dissociation of GDP from $G_{\alpha_i}$ (8–10). The AGS3 GPRs also compete with $G_{\beta\gamma}$ for interaction with $G_{\alpha_i}$ (8). In the context of $G_{i}$ heterotrimers, such an effect would lead to the generation of a $G_{\alpha_i}\mathrm{GDP}/AGS3$ complex with “release” of $G_{\beta\gamma}$ (6, 8). Indeed, in the protein interaction assays using heart lysates, $G_{\beta\gamma}$ subunits were not

**Fig. 10.** Influence of $G_{\alpha_i}$ and $G_{\alpha_i}$ on subcellular distribution of AGS3-SHORT and AGS3-LONG. COS-7 cells were transfected with pcDNA3 (Vector), pcDNA3-$G_{\alpha_i}$ (7.5 μg), pcDNA3-$G_{\alpha_i}$ (7.5 μg), pcDNA3-AGS3-SHORT-1 (2.5 μg), pcDNA3-AGS3-LONG (2.5 μg), or pcDNA3-G-protein plus pcDNA3, pcDNA3-AGS3-SHORT, or pcDNA3-AGS3-LONG. In each transfection, the total amount of plasmid used was 10 μg. Cells were harvested 48 h after transfection and processed as described under “Experimental Procedures.” A 100,000 g pellet and supernatant were prepared for each sample, and 20 μg of membrane protein was loaded in each lane. Membrane transfers were immunoblotted with PEP22 antisera. Blots were also stripped and reprobed with $G_{\alpha_i}$ in heart cell lysates (Fig. 10).
associated with the GST-AGS3-SHORT-Gαi complex. The interaction of AGS3 with Gαi/Gαt may also play a regulatory role in G-protein activation by G-protein-coupled receptors. The GPR domains of AGS3 actually block rhodopsin activation of transducin and also prevent the interaction of Gi proteins with G-protein-coupled receptors (9, 10). Alternatively, the Gαi(GDP)AGS3 complex may exist independent of Gbg and subserve unique regulatory functions within the cell, where it might receive input from a putative guanine nucleotide exchange factor other than a G-protein-coupled receptor. AGS3 is actually just one of several proteins that regulate the activation state of G-protein independent of the receptor. Such proteins may be key players in signal integration and the dysfunctional signaling events observed in pathophysiological settings.

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Identification of a Truncated Form of the G-protein Regulator AGS3 in Heart That Lacks the Tetratricopeptide Repeat Domains
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