INTERACTION OF AGS3 WITH LKB1, A SERINE-THREONINE KINASE
INVOLVED IN CELL POLARITY AND CELL CYCLE PROGRESSION:
PHOSPHORYLATION OF THE GPR-MOTIF AS A REGULATORY MECHANISM
FOR THE INTERACTION OF GPR MOTIFS WITH Giα


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ABSTRACT

Activator of G-protein signaling 3 (AGS3) has a modular domain structure consisting of seven tetratricopeptide repeats (TPRs) and four G protein regulatory (GPR) motifs. Each GPR motif binds to the α subunit of Gi/Go (Giα > Goα) stabilizing the GDP-bound conformation of Gα and apparently competing with Gβγ for GαGDP binding. As an initial approach to identify regulatory mechanisms for AGS3 – G-protein interactions, a yeast two-hybrid screen was initiated using the TPR and linker region of AGS3 as bait. This screen identified the serine/threonine kinase LKB1, which is involved in the regulation of cell cycle progression and polarity. Protein interaction assays in mammalian systems using transfected cells or brain lysate indicated the regulated formation of a protein complex consisting of LKB1, AGS3 and G-proteins. The interaction between AGS3 and LKB1 was also observed with orthologous proteins in Drosophila where both proteins are involved in cell polarity. LKB1-immunoprecipitates from COS7 cells transfected with LKB1 phosphorylated the GPR domains of AGS3 and the related protein LGN, but not the AGS3-TPR domain. GPR domain phosphorylation was completely blocked by a consensus GPR motif peptide and placement of a phosphate moiety within a consensus GPR motif reduced the ability of the peptide to interact with G-proteins. These data suggest that phosphorylation of GPR domains may be a general mechanism regulating the interaction of GPR-containing proteins with G-proteins. Such a mechanism may be of particular note in regards to the localized signal processing in the plasma membrane involving G-protein subunits and/or intracellular functions regulated by heterotrimeric G-proteins that occur independent of a typical G-protein coupled receptor.
AGS3 was identified in a functional screen for receptor-independent activators of G-protein signaling (1,2). Surprisingly, the activation of G-protein signaling in the functional screen was independent of nucleotide exchange on the Gα subunit suggesting unexpected mechanisms for regulating the activation state of heterotrimeric G-proteins. AGS3 interacts with G-proteins (Gi/Go) via its four G-protein regulatory (GPR) or GoLoco motifs, each of which interact with Gα (Gi > Go) and stabilize the GDP-bound conformation of Gα (3). The GPR motif is also found in other proteins including LGN, RGS12, RGS14, Rap1GAP, Pcp2 and G18.1b (1,4). AGS3 also contains seven tetratricopeptide repeats (TPR) in the first half of the protein, and these domains may serve as a regulatory domain for the GPR–G-protein interaction or they may target the protein to different microdomains within the cell (5). A similar motif structure is found in the AGS3-related protein LGN in mammals (6,7) as well as in the AGS3/LGN ortholog Pins in D. melanogaster, which is a key determinant of cell polarity (8-12). A role for GPR- containing proteins and G-proteins in cell polarity is also suggested by studies in C. elegans (13, 14).

AGS3 and other accessory proteins—proteins distinct from receptors, G-proteins and effectors—may influence receptor-mediated signaling events and/or mediate signal input to G-proteins independent of a G-protein coupled receptor. Such proteins may also serve as alternative binding partners for G-protein subunits independent of heterotrimer formation (1,2,15) and the existence of these accessory proteins suggest unexpected functional roles for G proteins within the cell. As an initial approach to define the
cellular control mechanisms for AGS3-G-protein interactions, we sought to identify binding partners for the TPR domains of AGS3.

We isolated several candidate AGS3-TPR interacting proteins in a yeast two-hybrid screen, one of which corresponded to the carboxyl terminal 107 amino acids of LKB1, also known as Serine/Threonine Kinase 11 (S/TK11) (16,17). Loss of LKB1 is implicated in Peutz-Jeghers Syndrome (PJS), a rare, inherited intestinal polyposis syndrome (16-18), and it is actually the mammalian counterpart of the *C. elegans* gene *par-4* (19), which is a member of a group of polarity determining genes during embryogenesis in both *C. elegans* and *Drosophila* (20,21). Significantly, LKB1 phosphorylates AGS3 in its GPR domain and this was completely blocked by a consensus GPR motif peptide. Placement of a phosphate moiety within a consensus GPR motif markedly reduced the ability of the peptide to interact with G-proteins suggesting that phosphorylation of GPR motifs may be a general mechanism regulating the interaction of GPR-containing proteins with G-proteins. Such a mechanism may be of particular note in regards to the localized signal processing in the plasma membrane involving G-protein subunits and/or intracellular functions regulated by heterotrimeric G-proteins that occur independent of a typical G-protein coupled receptor.
EXPERIMENTAL PROCEDURES

Materials- Yeast strains pre-transformed with prey libraries, Anti c-Myc monoclonal antisera and KC-8 chemically competent cells were obtained from Clontech (Palo Alto, CA). Bait vector pGBKT7 and yeast strains Y187 and AH109 were kindly provided by Dr. Tim McQuinn (Medical University of South Carolina). $\gamma^{32}$P-ATP and $^{32}$P-orthophosphate were obtained from NEN (Boston, MA) Sodium orthovanadate and RNAse A were obtained from Sigma (St. Louis, MO). Okadaic acid was obtained from Calbiochem (San Diego, CA). pMAL-c2x and amylose-agarose beads were obtained from New England Biolabs (Boston, MA). Other materials were obtained as described elsewhere (3,7).

Yeast two-hybrid screening - AGS3-TPR (Met$^1$-Ile$^{462}$) was generated by PCR. Restriction digested PCR products were subcloned into pGBKT7 to generate the TPR bait construct. TPR and empty pGBKT7 vector were transformed into AH109 by the lithium acetate method. Expression of bait fusion proteins was confirmed by immunblotting with Anti c-Myc. Basal activity of bait strains was assayed by nutritional selection. AH109 yeast strains expressing TPR as bait were mated with Y187 yeast strains expressing an 11-day old mouse embryo cDNA library by following the manufacturer’s protocol. The mated yeast culture was plated onto 120 quadruple dropout (QDO) (Trp- Leu- His- Ade-) plates, which were then incubated at 30°C for 7 days. β-galactosidase activity was screened using the colony-lift filter assay according to the manufacturer’s directions using diploid p53/SV40large T antigen interaction (diploid strain PJ69-2A[pVA3-1] x Y187[pTD1-1])
as a positive control as supplied by the manufacturer. Yeast plasmid DNA was isolated and used to transform competent KC-8 *E. coli* cells. Transformants containing the prey vector were selected by plating onto M9 Leu\(^{-}\) plates. Plasmids isolated from KC-8 transformants were transformed into XL1-Blue *E. coli* cells for further processing and retransformation of yeast strains.

**Immunoprecipitation and cell labeling** Confluent 100 mm dishes of COS7 cells were transfected with either 10µg empty vector (pcDNA3), 5µg pcDNA3::AGS3 + 5µg empty vector, or 5µg pcDNA3::AGS3 + 5µg pcDNA3::LKB1 (mouse). After 24 hours, cells were lysed in NP40 lysis buffer and incubated on ice 1 h. The lysate was centrifuged at 100,000 x g for 30 min at 4°C, and was pre-cleared with Gamma-Bind sepharose (Pharmacia). The pre-cleared lysates (1mg protein) were incubated with 5 µg anti-LKB1 (Upstate Biotechnology, Inc., Lake Placid, NY) for 12-18 hrs at 4°C. Gamma-Bind sepharose was added and incubation continued for 30 min. The resin was pelleted and used for kinase assays are washed 3 times with NP40 lysis buffer resuspended in 5 X protein sample buffer and placed in a boiling water bath for 3 minutes followed by SDS-PAGE and immunoblotting with AGS3-specific (PEP32) (3) and LKB-specific (P6) (22) antisera. PC12 cells were labeled with \(^{32}\)P-orthophosphate (8500-9120 Ci/mmol) according to Kang et al. except that phosphate-free DMEM was used (23).

For experiments with the *Drosophila* proteins, 3.5-7 hour collection of wild-type *Drosophila* embryos was dechorionated and lysed by dounce homogenization in ice-cold embryo lysis buffer (25mM Tris-HCl pH 8.0, 27.5mM NaCl, 20mM KCl, 25mM sucrose, 10mM EDTA, 10mM EGTA, 1mM PMSF, 1mM DTT, 10% glycerol, 0.1% NP40,
supplemented with protease and phosphatase inhibitors). The resultant lysate was centrifuged at 14000 rpm in a 4°C bench-top centrifuge and the supernatants used for immunoprecipitation. Each immunoprecipitation (1:60 dilution of antisera) used lysate corresponding to 150 ul of packed embryos in a total volume of 300 ul. Immunoblots were performed with mouse-anti-LKB1 (1:200) or rabbit anti-Pins (1:100).

**Kinase assay** —LKB1 immunoprecipitates were washed 3 times with NP40 lysis buffer and 3 times with kinase buffer A (50 mM Tris pH 7.5, 0.1% β-mercaptoethanol, 0.1 mM EGTA, 10 mM MnCl₂, 0.5 μM okadaic acid) (17). The Gamma-Bind sepharose was then resuspended in kinase buffer A containing 10 μM γ³²P-ATP (1000cpm/pmol) and 1 μM purified GST fusion protein. Reactions were incubated at 30°C for one hour. The Gamma-Bind sepharose beads were pelleted, the supernatant removed and incubated with glutathione sepharose (Pharmacia) for 30 min at 24°C to isolate the GST fusion proteins. The glutathione sepharose was pelleted and washed 3 times with kinase buffer A, resuspended in 5 X protein sample buffer and placed in a boiling water bath for 3 minutes followed by SDS-PAGE and autoradiography.
RESULTS AND DISCUSSION

Although AGS3 clearly interacts with G-proteins, and the GPR motifs in AGS3 and other GPR-containing proteins stabilize the GDP-bound conformation of Gα, only a subpopulation of AGS3 and G-proteins are associated with each other in brain lysates (3) and the two proteins exhibit minimal overlap in terms of their subcellular distribution (5). These data suggest that the interaction between AGS3 and G-proteins is a regulated event. As part of a broader strategy to address this issue and define the role of AGS3-G-protein interactions in cellular function, we used a protein interaction screen to identify binding partners for the TPR and linker region of AGS3. A yeast two-hybrid screen of a mouse 11-day old embryonic cDNA library using AGS3-TPR (Met^1-Ile^462) as bait yielded several candidate AGS3 binding partners. The screen was run with high stringency by directly using quadruple dropout selection (Trp^-Leu^-His^-Ade^-) followed by a secondary selection for colonies that exhibited strong β-gal activity within 30 minutes. Sixteen cDNAs were isolated, five of which encoded DNA binding proteins or proteins involved in regulation of transcription or translation. Of the remaining 11, one cDNA clone encoded an extracellular protein and 5 encoded previously unidentified proteins or proteins of unknown function. The remaining cDNA clones encoded portions of murine robo-1, an axonal guidance receptor during CNS development (24); microtubule/actin crosslinking factor (MACF, also known as ACF7), a member of the plakin family implicated in epithelial and neuronal polarity (25); MARCKS-like protein, a regulator of actin dynamics, migration, and neuronal development (26); and LKB1/STK11 (27). LKB1 and the Drosophila AGS3/LGN ortholog PINS are both involved in various
aspects of cell polarity and development. As a tertiary screen to select for proteins of potential interest, we asked if GST fusion proteins of each of these cDNA clones interacted with full length AGS3 in brain lysates. Only GST-MACF and GST-LKB1 effectively pulled down AGS3 from rat brain lysates. We first focused our effort on LKB1 as the LKB1 ortholog in *C. elegans* was previously identified as a PAR gene (19) involved in asymmetric division of *C. elegans* embryos. AGS3/LGN orthologs or proteins containing GPR motifs are also involved in similar events in *Drosophila* and *C. elegans*.

The cDNA clone encoding LKB1 contained the last 107 amino acids of the coding region of LKB1. The interaction of AGS3-TPR (Met<sup>1</sup>-Ile<sup>462</sup>) with LKB1 in the yeast two-hybrid screen required amino acids Asp<sup>338</sup>-Ile<sup>462</sup> in the AGS3 coding region that connects the TPR and GPR domains. Additional regions of AGS3 and LKB1 may also interact with each other in the context of the full-length proteins. We then asked if the interaction between the carboxyl terminus of LKB1 and AGS3 was observed in a mammalian system using a GST fusion protein of the LKB1 fragment isolated in the yeast two-hybrid screen. LKB1-CT (Asp<sup>330</sup>-Gln<sup>436</sup>) effectively interacted with endogenous, full length AGS3 in rat brain lysates (Fig. 1). Interestingly, this complex also contained Giα subunits, which is likely due to an interaction of G-proteins with the GPR motifs of AGS3 (3). The presence of G-proteins in this complex was nucleotide-dependent in that it was not observed in the presence of the nonhydrolyzable GTP analog GTPγS, which is consistent with the demonstrated preference of GPR motifs for the GDP-bound conformation of Giα (1,3,28). The interaction of AGS3 itself with LKB1 was not influenced by guanine nucleotides.
The LKB1-AGS3 interaction was further addressed with the full-length proteins in the intact cell. Co-transfection of cDNAs encoding full length AGS3 and LKB1 in COS7 cells and subsequent immunoprecipitation with LKB1 antisera resulted in co-immunoprecipitation of AGS3 (Fig 1B). This interaction was specific for LKB1 as immunoprecipitation with LKB1 antisera from cells transfected with AGS3 alone did not co-immunoprecipitate AGS3 (Fig 1B). In contrast to the results obtained with the GST-LKB1-CT (Asp$^{330}$-Gln$^{436}$) fusion protein in which Giα was brought down with the LKB-CT—AGS3 complex from brain lysates, Giα was not found in the coimmunoprecipitation complex of the full-length proteins suggesting that LKB1 may process incoming signals to regulate the interaction between AGS3 and G-proteins or target the protein to a microdomain where G-proteins are inaccessible.3

To provide further evidence for a functional interaction between LKB1 and AGS3, we asked if the interaction was evolutionarily conserved in Drosophila. Drosophila LKB1 (DmLKB1) coimmunoprecipitated with the AGS3 ortholog Pins (Partner of Inscuteable) and; conversely, Pins coimmunoprecipitated with DmLKB in Drosophila embryo lysates, indicating an interaction of the full-length Drosophila proteins (Fig 1C). In both the COS7 transfectants and the Drosophila embryos only a subpopulation of AGS3 (or Pins) was actually complexed with LKB1 following immunoprecipitation. This may reflect the affinity of the interaction, stoichiometric considerations and/or the regulation of the interaction by an as yet undefined signal(s). The AGS3 ortholog in Drosophila plays a critical role in cell polarity that apparently also involves heterotrimeric G proteins (8-12). The LKB1 ortholog in Drosophila was also recently identified in a genetic screen for defects in oocyte and epithelial cell polarity.
The demonstration of an interaction between LKB1 and AGS3/LGN orthologs in Drosophila provide additional evidence for functionality of this interaction and suggest a role for this interaction in the regulation of cell polarity and cell division.

The 180 amino acid kinase domain (Lys^{44}-Pro^{314}) of LKB1 is upstream of the region interacting with AGS3 (Fig 1). To determine if AGS3 is phosphorylated by LKB1, we performed in vitro kinase assays using LKB1 immunoprecipitated from LKB1-transfected COS7 cells and purified GST-AGS3 fusion proteins as substrate (Fig. 2A). The region of AGS3 containing GPR motifs was specifically phosphorylated by LKB1, whereas the TPR-linker domain of AGS3 was not (Fig. 2A). The specific phosphorylation of the region of AGS3 containing the GPR motifs is of particular interest as this region of the protein serves as a potential scaffold for G-protein $\alpha$ subunits (3). There are 24 serines/threonines in the GPR domain of AGS3, 16 of which are found in the GPR motifs themselves. All known GPR motifs contain 1-3 serines/threonines. LKB1 also phosphorylated the GPR domain found in the AGS3-related protein LGN and this is of particular interest as both LKB1 and LGN regulate the progression of the cell cycle (7,29,30). $^{32}$P-orthophosphate labeling experiments in PC12 cells, which express endogenous AGS3, followed by immunoprecipitation with AGS3-specific antisera indicate that AGS3 is indeed phosphorylated (Fig. 2C).

Although we do not as yet know the precise site of phosphorylation of AGS3-GPR by LKB1, the phosphorylation of the GPR domain was completely blocked by a consensus GPR peptide (Fig. 2B). The action of the GPR peptide is specific as a scrambled GPR peptide containing the same residues in a different order was ineffective (Fig 2B). These data suggest that the GPR motif is either itself the site of
phosphorylation and docks within the active site of LKB1 or it is an additional anchor for protein interaction. Peptidomimetics derived from the GPR motif may actually be a path for the development of LKB1 inhibitors.

As the GPR peptide effectively blocked AGS3-GPR phosphorylation by LKB1 immunoprecipitates, we asked if phosphorylation within the GPR motif could potentially influence the interaction of AGS3 with G-proteins. We initially addressed this possibility by placing a phosphate moiety on a serine found in the core of the GPR motif. A 28 amino acid peptide encompassing the core consensus GPR motif effectively inhibits the interaction of GPR-containing proteins with Gi\(\alpha\) and also mimics the action of GPR-containing proteins by inhibiting the binding of GTP\(\gamma\)S to G-protein (28). This action of the GPR peptide involves discrete residues within the GPR motif (4,31), mutation of which leads to a loss of activity as indicated for the Q22A peptide (31) (Fig 3A). Phosphorylation of a serine residue within the GPR motif immediately downstream of the invariant residue Gln15 markedly decreased its ability to interact with Gi\(\alpha\) and to inhibit GTP\(\gamma\)S binding to Gi\(\alpha\) (Fig 3A,B).

LKB1 and other serine/threonine kinases may exert a regulatory influence on the interaction of G protein \(\alpha\) subunits with GPR motif-containing proteins by phosphorylation of residues within the GPR motif. GPR motifs are found in several proteins involved in signal propagation including LGN, RGS12, RGS14, Rap1GAP, Pcp2 and G18.1b (1,4). A recent report also suggested that phosphorylation near the GPR motif of RGS14 may influence GPR-Gi\(\alpha\) interaction (32). Such a mechanism may allow the discrete regulation of G-protein signaling in specific subcellular compartments that occur independent of a G-protein coupled receptor. The localized regulation of such events is a
signature mechanism for the determination of cell polarity and asymmetric cell division observed in stem cells during tissue development.
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FOOTNOTES


3 Blumer J.B. and S.M. Lanier, unpublished observations.

4 Hall A.  (Personal communication) AGS3 was also isolated in a yeast two-hybrid screen using LKB1 as bait and both proteins reported to be involved in astrocyte polarity.
LITERATURE CITED

FIGURE LEGENDS

Figure 1. Interaction of LKB1 with AGS3. (A) Rat brain (2 mg) lysate was preincubated with 30 µM GDP or 30 µM GTPγS/25 mM MgCl₂ at 24°C for 30 minutes. Lysates were then incubated with 500 nM GST or GST-LKB1-CT (Asp³³⁰-Gln⁴³⁶) for one hour at 24°C. Protein complexes were captured by glutathione sepharose beads and analyzed by immunoblotting following SDS-PAGE. Membrane transfers were first blotted with AGS3 antisera then stripped and reprobed with Giα₃ antisera. The input lane contains 1/10 of the lysate volume used for each interaction assay. The data are representative of 2 experiments. (B) LKB1 co-immunoprecipitates AGS3. COS7 cells transiently transfected with empty vector (V), pcDNA3::AGS3, or pcDNA3::AGS3 + pcDNA3::LKB1 were lysed in NP40 buffer prior to immunoprecipitation with LKB1-specific antisera and immunoblotting with AGS3-specific (PEP32) and LKB-specific (P6) antisera. (C) Drosophila AGS3 ortholog Pins interacts with Drosophila LKB1 in embryo lysates. Drosophila embryo lysates were immunoprecipitated with DmLKB1 or Pins antisera and association of these two proteins was evaluated by SDS-PAGE and immunoblotting for Pins (top panel) and DmLKB1 (bottom panel). The input lane represents 1/40 of the lysate volume used for each immunoprecipitation.

Figure 2. LKB1 phosphorylates the GPR domain of AGS3. (A) Top panel - COS7 cells were transfected with 10 µg pcDNA3::LKB1 or empty vector for 24 hours prior to immunoprecipitation and immunoblotting with LKB1 antisera. Bottom panel - Purified GST fusion proteins (1 µg per lane) were subjected to SDS-PAGE and Coomassie blue
staining. Right panel - LKB1 immunoprecipitates from LKB1 transfected COS7 cells were incubated with 1 µM GST, GST-AGS3-TPR (Met\(^1\)-Ile\(^{462}\)), or GST-AGS3-GPR (Pro\(^{463}\)-Ser\(^{650}\)) in the presence of \(\gamma^{2}\)P-ATP for 1 hour at 30°C as described in “Experimental Procedures”. GST fusion proteins were purified with a glutathione resin prior to SDS-PAGE and autoradiography. The data are representative of 3 experiments.

(B) LKB1 kinase assays were performed as described in “Experimental Procedures” with GST or GST-AGS3-GPR (Pro\(^{463}\)-Ser\(^{650}\)) fusion proteins in the presence or absence of a consensus GPR peptide (TMGEEDFFDLLAKSQSKRDDQRVDLAG) or a scrambled control peptide (TMGDDQRLAKSQSKRMEDFFVDLAG) at a final concentration of 100 µM. GST fusion proteins were purified with glutathione sepharose, washed and subjected to SDS-PAGE and autoradiography. Data are representative of 2 experiments.

(C) PC12 cells, which express endogenous AGS3, were labeled with \(^{32}\)P-orthophosphate 3 hours prior to cell lysis (~ 500 µg protein), immunoprecipitation with AGS3 antisera, followed by SDS-PAGE and autoradiography. Parallel unlabeled samples (~ 500 µg protein) were used for immunoblotting with anti-AGS3 antisera to confirm expression and immunoprecipitation. Data are representative of 2 experiments.

**Figure 3. Influence of GPR phosphorylation on G-protein interaction.** (A) Protein interaction assays were performed as described in “Experimental Procedures” using 75 nM Gi\(\alpha\)1 and 300 nM GST-AGS3 in the presence of 10 µM GDP. Peptide concentration = 10 µM. Similar results were obtained in 3 separate experiments. The input lane represents 1/10 of the total volume in each interaction assay. (B) GTP\(\gamma^{35}\)S (500 nM) binding to Gi\(\alpha\) (100 nM) was measured after incubation with increasing concentrations
of control GPR consensus peptide and a phosphorylated Ser16 GPR peptide (phosphorylation site indicated by asterisk) as described in “Experimental Procedures.” Protein interaction assays and GTPγS binding assays were performed as described (27). Data are expressed as the percent of specific binding (~0.5 pmol) observed in the absence of added peptide and are expressed as the mean +/- S.E of two experiments with duplicate determinations.
Figure 2

A

IP: LKB
IB: LKB
vector LKB1

GST GST
GST TPR GPR

Coomassie Blue

autorad

B

vector LKB

GST TPR GPR
GST TPR GPR

GST-GPR

C

PC12
(endogenous AGS3)

IP: AGS3
autorad IB: AGS3

AGS3
Figure 3

Panel A: Western blot analysis showing the expression levels of Giα1 and GST-AGS3 proteins. The arrow indicates the Giα1 protein band. The lanes are labeled as follows: Input, GST, Vehicle, GPR, GPR (Q22A), and GPR (PhosphoS16).

Panel B: Graph showing the GTPγS binding (% control) against the logarithm of the peptide concentration (M). Two curves are plotted: one for the control GPR peptide and another for the phosphorylated GPR peptide. The graph includes error bars indicating the variability of the data.
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