Interaction of Activator of G-protein Signaling 3 (AGS3) with LKB1, a Serine/Threonine Kinase Involved in Cell Polarity and Cell Cycle Progression

PHOSPHORYLATION OF THE G-PROTEIN REGULATORY (GPR) MOTIF AS A REGULATORY MECHANISM FOR THE INTERACTION OF GPR MOTIFS WITH Gα

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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 G/Gα (Gα > Gα) stabilizing the GDP-bound conformation of Gα and apparently competing with Gβγ for Gα-GDP binding. As an initial approach to identify regulatory mechanisms for AGS3-G-protein interactions, we sought to identify binding partners for the TPR domains of AGS3 and other accessory proteins (proteins distinct from peptides) that can bind to G-proteins independently of a G-protein coupled receptor. Such proteins may also serve as alternative binding partners for G-protein subunits independently of heterotrimeric G-proteins. AGS3 interacts with G-proteins (G/Gα) via its four G-protein regulatory (GPR) or GoLoco motifs, each of which interacts with Gα (Gα > Gα) and stabilizes the GDP-bound conformation of Gα (3). The GPR motif is also found in other proteins including LGN, RGS12, RGS14, Rap1GAPII, Pcp2, and G18.1b (1, 4). AGS3 also contains seven tetratricopeptide repeats (TPRs) 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 Drosophila 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 Caenorhabditis 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 independently of a G-protein coupled receptor. Such proteins may also serve as alternative binding partners for G-protein subunits independently of heterotrimer formation (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 (G/Gα) via its four G-protein regulatory (GPR) or GoLoco motifs, each of which interacts with Gα (Gα > Gα) and stabilizes the GDP-bound conformation of Gα (3). The GPR motif is also found in other proteins including LGN, RGS12, RGS14, Rap1GAPII, Pcp2, and G18.1b (1, 4). AGS3 also contains seven tetratricopeptide repeats (TPRs) 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 Drosophila 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 Caenorhabditis elegans (13, 14).

AgS31 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 (G/Gα) via its four G-protein regulatory (GPR) or GoLoco motifs, each of which interacts with Gα (Gα > Gα) and stabilizes the GDP-bound conformation of Gα (3). The GPR motif is also found in other proteins including LGN, RGS12, RGS14, Rap1GAPII, Pcp2, and G18.1b (1, 4). AGS3 also contains seven tetratricopeptide repeats (TPRs) in the first half of the peptide, 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 Drosophila 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 Caenorhabditis 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 independently of a G-protein coupled receptor. Such proteins may also serve as alternative binding partners for G-protein subunits independently of heterotrimer formation (1, 2, 15), and the existence of these accessory proteins suggests 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 GPR 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 (STK11) (16). Loss of LKB1 is implicated in Peutz-Jeghers syndrome, a rare inherited intestinal

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1 The abbreviations used are: AGS3, activator of G-protein signaling 3; TPR, tetratricopeptide repeat; GPR, G-protein regulatory; Pins, Partner of Inscuratcule; Ade, adenine; GST, glutathione S-transferase; MACF, microtubule/actin cross-linking factor; STK11, serine/threonine kinase 11; CT, carboxyl terminus; GTP, guanosine 5′-O-(3′-thiotriphosphate; Dm, Drosophila melanogaster; RGS, regulator of G-protein signaling; GAP, GTPase-activating protein.

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polyposis syndrome (16–18), and LKB1 is actually the mammalian counterpart of the C. elegans gene par-4, which is a member of a group of polarity-determining genes during embryogenesis in both C. elegans and Drosophila (19–21). 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 regard to the localized signal processing in the plasma membrane involving G-protein subunits and/or intracellular functions regulated by heterotrimeric G-proteins that occur independently of a typical G-protein coupled receptor.

EXPERIMENTAL PROCEDURES

Materials—Yeast strains pretransformed with prey libraries, c-Myc monoclonal antisera, and KC-8 chemically competent cells were obtained from Clontech (Palo Alto, CA). Bait vector pGBKTK7 and yeast strains Y187 and AH109 were kindly provided by Dr. Tim McQuinn (Medical University of South Carolina), (γ-32P)ATP and (32P)orthophosphate were obtained from PerkinElmer Life Sciences. Sodium orthovanadate and RNase A were obtained from Sigma. Okadaic acid was obtained from Calbiochem. pMal-c2x and amylase-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 (Met1–Ile163) was generated by PCR. Restriction enzyme-digested PCR products were subcloned into pGBKTK7 to generate the TPR bait construct. TPR and empty pGBKTK7 vector were transformed into AH109 by the lithium acetate method. Expression of bait fusion proteins was confirmed by empty pGBKT7 vector were transformed into AH109 by the lithium acetate method. Expression of bait fusion proteins was confirmed by immunoblotting 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 (Trp−Leu−His−Ade−) plates that 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 diethyl p-53/SV40 large T antigen interaction (diplodiploid strain PJ69-4A[pV3-1] × Y187[pTD1-1]) as a positive control as supplied by the manufacturer. Yeast plasmid DNA was isolated and used to transform competent KC-8 Escherichia 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 transformation of yeast strains.

Immunoprecipitation and Cell Labeling—100-mm dishes of confluent COS7 cells were transfected with either 10 μg of empty vector (pcDNA3), 5 μg of pcDNA3:AGS3 + 5 μg of empty vector, or 5 μg of pcDNA3:AGS3 + 5 μg of pcDNA3::LKB1 (mouse). After 24 h, cells were lysed in Nonidet P-40 lysis buffer and incubated on ice for 1 h. The lysate was centrifuged at 100,000 × g for 30 min at 4 °C and precleared with GammaBind Sepharose (Amersham Biosciences). The precleared lysates (1 mg of protein) were incubated with 5 μg of anti-LKB1 (Upstate Biotechnology, Inc., Lake Placid, NY) for 12–18 h at 4 °C. GammaBind Sepharose was added, and incubation continued for 30 min. The resin was pelleted and used for kinase assays. Samples were washed three times with Nonidet P-40 lysis buffer, resuspended in 5× protein sample buffer, and placed in a boiling water bath for 3 min 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 Go, 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 (Met1–Ile163) 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-galactosidase activity within 30

FIG. 1. Interaction of LKB1 with AGS3. A, rat brain (2 mg) lysate was preincubated with 30 μM GDP or 30 μM GTP·S, 25 mM MgCl2 at 24 °C for 30 min. Lysates were then incubated with 500 nM GST or GST-LKB1-CT (Asp351–Gln462) for 1 h 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 antiseraum and then stripped and reprobed with Gβ5, antisem. The Input lane contains 1/3 of the lysate volume used for each interaction assay. The data are representative of two experiments. B, LKB1 co-immunoprecipitates AGS3. COS7 cells transiently transfected with empty vector (V), pcDNA3:AGS3, or pcDNA3:AGS3 + pcDNA3::LKB1 were lysed in Nonidet P-40 buffer prior to immunoprecipitation with LKB1-specific antiseraum and immunoblotting with AGS3-specific (PEP92) and LKB-specific (P6) antisera. C, Drosophila LKB1 ortholog interacts with Drosophila LKB1 in embryo lysates. Drosophila embryo lysates were immunoprecipitated with pcDNA3::LKB1 or Pins antisem 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 1/3 of the lysate volume used for each immunoprecipitation. IP, immunoprecipitation.
proteins were purified with a glutathione resin prior to SDS-PAGE and LKB1 kinase assays were performed as described under "Experimental Procedures." A protein interaction assay was performed as described under "Experimental Procedures" using 75 nM G\(_{\alpha}\) and 300 nM GST-AGS3 in the presence of 10 \(\mu\)M GDP. Peptide concentration = 10 \(\mu\)M. Similar results were observed in three separate experiments. The Input lane represents \(\%\) of the total volume in each interaction assay. B: GTP\(_\gamma\)S (500 nm) binding to G\(_{\alpha}\) (100 nm) was measured after incubation with increasing concentrations of control GPR consensus peptide and a phosphorylated Ser\(_{15}\) (PhosphoS15) GPR peptide as described under "Experimental Procedures." Protein interaction assays and GTP\(_\gamma\)S binding assays were performed as described previously (26). Data are expressed as the percentage of specific binding (\(\pm\) 0.5 pmol) observed in the absence of added peptide and are expressed as the mean \(\pm\) S.E. of two experiments with duplicate determinations.

The cDNA clone encoding LKB1 contained the last 107 amino acids of the coding region of LKB1. The interaction of AGS3-GPR (Pro\(_{463}\)-Ile\(_{462}\)) with LKB1 in the yeast two-hybrid screen required amino acids Asp\(_{330}\)-Ile\(_{462}\) in the AGS3 coding region that connects the TPR and GPR domains (2). Additional regions of AGS3 and LKB1 may also interact with each other in the context of the full-length proteins. We then asked whether 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\(_{330}\)-Gln\(_{436}\)) effectively interacted with endogenous, full-length AGS3 in rat brain lysates (Fig. 1). Interestingly this complex also contained G\(_{\alpha}\) 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 GTP\(_\gamma\)S, which is consistent with the demonstrated preference of G-proteins for the GDP-bound conformation of G\(_{\alpha}\) (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 antiserum resulted in co-immunoprecipitation of AGS3 (Fig. 1B). This interaction was specific for LKB1 as immunoprecipitation with LKB1 antiserum 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 G\(_{\alpha}\) was brought down with the LKB1-CT-AGS3 complex from brain lysates, G\(_{\alpha}\) was not found in the co-immunoprecipitation 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.

To provide further evidence for a functional interaction between LKB1 and AGS3, we asked whether the interaction was evolutionarily conserved in Drosophila. Drosophila LKB1 (DmLKB1) co-immunoprecipitated with the AGS3 ortholog Pins, and conversely Pins co-immunoprecipitated with DmLKB1 in Drosophila embryo lysates, indicating an interaction between LKB1 and Pins. 

* M. L. Bernard, J. B. Blumer, and S. M. Lanier, unpublished observations.

** J. B. Blumer, Y. K. Peterson, and S. M. Lanier, unpublished observations.

AGS3 was also isolated in a yeast two-hybrid screen using LKB1 as bait, and both proteins were reported to be involved in astrocyte polarity (A. Hall, personal communication).
tion 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 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 (21). The demonstration of an interaction between LKB1 and AGS3/LGN orthologs in (21). The demonstration of an interaction between LKB1 and AGS3/LGN orthologs in

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