Receptor-independent Activators of Heterotrimeric G-protein Signaling Pathways*

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Heterotrimeric G-protein signaling systems are activated via cell surface receptors possessing the seven-membrane span motif. Several observations suggest the existence of other modes of stimulus input to heterotrimeric G-proteins. As part of an overall effort to identify such proteins we developed a functional screen based upon the pheromone response pathway in *Saccharomyces cerevisiae*. We identified two mammalian proteins, AGS2 and AGS3 (activators of G-protein signaling), that activated the pheromone response pathway at the level of heterotrimeric G-proteins in the absence of a typical receptor. β-galactosidase reporter assays in yeast strains expressing different Go subunits (Gpa1, Gsa, G(Gpa1(1–41)), G(GDpa1(1–41)), G(Ga1(1–41))) indicated that AGS proteins selectively activated G-protein heterotrimers. AGS3 was only active in the Gsa3 genetic backgrounds, whereas AGS2 was active in each of the genetic backgrounds except Gpa1. In protein interaction studies, AGS2 selectively associated with Gβγ, whereas AGS3 bound Go and exhibited a preference for GaGDP versus GaGTPγS. Subsequent studies indicated that the mechanisms of G-protein activation by AGS2 and AGS3 were distinct from that of a typical G-protein-coupled receptor. AGS proteins provide unexpected mechanisms for input to heterotrimeric G-protein signaling pathways. AGS2 and AGS3 may also serve as novel binding partners for Ga and Gβγ that allow the subunits to subserve functions that do not require initial heterotrimer formation.

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The seven-membrane span hormone receptor coupled to heterotrimeric G-proteins represents one of the most widely used systems for information transfer across the cell membrane. Signal processing via this system likely operates within the context of a signal transduction complex. Within such a signal transduction complex, there are likely accessory proteins (distinct from receptor, G-protein, and effectors) that participate in the formation of this complex and/or regulate signal transfer from receptor to G-protein. In addition, several reports suggest alternative modes of stimulus input to heterotrimeric G-proteins that do not require direct interaction of the G-protein with the seven-membrane span receptor itself. To identify such entities and to define putative components of such a signal transduction complex we initiated two broad experimental approaches (1–4). One strategy focused on a functional readout involving G-protein activation and was based upon initial observations in our laboratory concerning the transfer of signal from R to G (3, 4). This approach resulted in the partial purification and characterization of the NG10815 G-protein activator that directly increased GTPγS binding to brain G-protein in the absence of a receptor. To extend this body of work, we developed an expression cloning system in *Saccharomyces cerevisiae* that was designed to detect mammalian activators of the pheromone response pathway in the absence of a G-protein-coupled receptor (5). The pheromone response pathway in *S. cerevisiae* incorporates a seven-membrane span receptor, a heterotrimeric G-protein, and a mitogen-activated protein kinase cascade that regulates mating behavior and growth (6). In this report, we present the identification and characterization of two proteins isolated in this functional screen. These proteins were termed AGS21 and AGS3 for activators of G-protein signaling.

EXPERIMENTAL PROCEDURES

**Generation and Screening of cDNA Libraries**—The NG108-15 cell line was propagated as described previously (4). mRNA was prepared from twenty 100-mm confluent plates of cells and was used to generate a cDNA library in the yeast expression vector pYES2 by standard procedures using reagents from Stratagene. The NG108-15 cDNA library was screened for activators of the pheromone response pathway as described (5). We used a his3 far1 yeast strain containing a genomic integration of the FUS1p-HIS3 reporter construct and lacking both the native pheromone response receptor Ste3 and Gpa1 Ga subunit. A plasmid carrying a modified mammalian Gaα2 subunit in which the amino terminus was replaced with the first 41 amino acids of Gpa1 was introduced into this strain. Essentially, the screen took advantage of the inducible expression of the library cDNAs by differential plating of transformants on selective medium.

**cDNA Analysis**—A rat brain cDNA library was screened with a 32P-labeled 43-mer oligonucleotide (5'–ATAAGGCCTGAAGAAATCCT- CATCAGGATGTTAGGCCC-3') derived from the AGS3 sequence cDNA isolated in the yeast screen. The longest brain AGS cDNA still contained a truncated reading frame, and the 5'-end was extended by 5'-RACE using Marathon-Ready cDNA (CLONTECH) from rat brain. Domain searches were performed by the GCG COMPARE/DOTPLOT PROFILESEARCH and MOTIFS commands via internet access to the simple modular architecture research tool (SMART) data bases (7). Multiple expectation maximization for motif elucidation version 2.2 (MEME), motif alignment and search tool (MAST), and profile searches were performed via web resources at the San Diego Supercomputer Center (8). Secondary structure analysis was performed with the GCG

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1 The abbreviations used are: AGS, activators of G-protein signaling; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-((thio)triphosphate; nt, nucleotide(s); GPR, G-protein regulatory; RACE, rapid amplification of cDNA ends.
were incubated with GST or GST
NG108-15 cDNA clones.

of Gi3 (1 to 50 dilution of sera) was incubated with 250
munoprecipitation, antiserum generated against the carboxyl terminus
prior to solubilization and denaturing gel electrophoresis (2). For im-

40
150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 60
trifuged, and the matrix was washed with 2

that promoted growth in a galactose-dependent manner (Fig. 1,

B, epistasis experiments with duplicate cDNA clones. B, interaction of AGS2 and AGS3 with G-proteins. AGS2 and the bioactive AGS3 peptide (74 amino acids) beginning at the first in frame methionine were generated as

GTPαS) = 10 μM, total volume = 250 μl) for 12 h at 4 °C. GDP = 10 μM, GTPγS = 19 μM
plus 5 mM MgCl2. Proteins were then adsorbed to glutathione matrix and
retained G-protein subunits identified by immunoblotting following
gel electrophoresis. Gαα was generated in S9F cells as an amino-termi-
nal His6-tagged protein and was detected using anti-Xpress antiserum (Invitrogen). Input, 20 μl of the incubation mixture. The results presented are representative of four separate experiments. The GST-AGS fusion protein was functionally similar to the original AGS3 isolate in

in yeast strains lacking Ste20 (yeast homolog

of G204A Gi

16(Gpa1(1–41)). In



3

of p21-activated kinases) or Ste4 (yeast homolog of Gβ), indicating that these proteins activated the pheromone response pathway at or near the level of G-protein (Fig. 1B). Efforts were focused on cDNAs #37 and 53. Immunoblot analysis indicated that neither cDNA altered the levels of Gα or Gβ subunits (data not shown). The selectivity of the two cDNAs for different

G-protein heterotrimeric was determined using yeast strains expressing Gpa1 (yeast Gα), Gαα, Gααα(Gpa1(1–41)), Gαβ(Gpa1(1–41)), and Gαγ(Gpa1(1–41), and Gααα(Gpa1(1–41)). In β-galactosidase reporter assays, cDNA #37 was active in each of the genetic backgrounds except

Gpa1, whereas cDNA #53 was only active in the Gααα and Gαγ genetic backgrounds (Fig. 2A). The preceding observations indicated that the activation of the pheromone response pathway by

cDNAs #37 and 53 depended upon the presence of hetero-

trimeric G-proteins and the composition of subunit isoforms. cDNAs isolated via this expression cloning system were there-
dependent upon the presence of hetero-

RESULTS AND DISCUSSION

To facilitate the identification of novel receptor-independent activators of heterotrimeric G-proteins, we developed an ex-

pression cloning system based upon the pheromone response pathway in S. cerevisiae. We used a yeast strain that lacked the

pheromone receptor and contained a modified mammalian G-

protein (Gαα) in place of the yeast G-protein Gpa1 (5, 11). The

yer strain was further modified to respond to activation of the

pheromone response pathway with a readout of growth (5). As

an initial source of such G-protein activators (4) we generated a

NG108-15 cDNA library in the galactose inducible yeast expression vector pYES2. Three rounds of transformation/ screening with different libraries yielded three distinct cDNAs that

promoted growth in a galactose-dependent manner (Fig. 1, A and B). Epistasis experiments indicated that cDNA #34, which was the weakest of the three in the yeast screen, acted downstream of Ste5 (a component of the yeast mitogen-activated protein kinase cascade) in the pheromone response pathway, and this protein will be described elsewhere. cDNAs #37 and 53 did not function in the null Ste5 genetic background and

were also inactive in yeast strains lacking Ste20 (yeast homolog of p21-activated kinases) or Ste4 (yeast homolog of Gβ), indicating that these proteins activated the pheromone response pathway at or near the level of G-protein (Fig. 1B). Efforts were focused on cDNAs #37 and 53. Immunoblot analysis indicated that neither cDNA altered the levels of Gα or Gβ subunits (data not shown). The selectivity of the two cDNAs for different G-protein heterotrimers was determined using yeast strains expressing Gpa1 (yeast Gα), Gαα, Gααα(Gpa1(1–41)), Gαβ(Gpa1(1–41), and Gαγ(Gpa1(1–41)). In β-galactosidase reporter assays, cDNA #37 was active in each of the genetic backgrounds except Gpa1, whereas cDNA #53 was only active in the Gααα and Gαγ genetic backgrounds (Fig. 2A). The preceding observations indicated that the activation of the pheromone response pathway by cDNAs #37 and 53 depended upon the presence of heterotrimeric G-proteins and the composition of subunit isoforms. cDNAs isolated via this expression cloning system were therefore named activators of G-protein signaling (AGS). cDNAs #37 and 53 were termed AGS2 and AGS3, respectively. AGS1, isolated from a human liver cDNA library (5), WT, wild type strain CY1316/1183 containing Gααα as described for the original yeast screen. Similar results were obtained in three experiments.

AGS2 (770 nt) encoded a protein identical to mouse Tcetx1, a
light chain component of the cytoplasmic motor protein dynein and the flagellar dynein inner arm (12, 13). Tctex1 also exists in the cell free of dynein where it may subserve as yet undefined roles in cellular signaling (14). Tctex1 physically associates with the G-protein-coupled receptor rhodopsin, the tyrosine kinase fyn, and a putative regulator of neurotransmitter release Doc2 (15–17). The functionality of AGS2 (Tctex1) in the yeast assay system also suggests a direct interaction with heterotrimeric G-proteins. This issue was addressed in protein interaction studies using a GST-AGS2 fusion protein and purified G-protein subunits. AGS2 did not interact with G\(\alpha\)z but directly bound brain G\(\beta\)\(\gamma\) (Fig. 2B). The bioactivity associated with AGS2 in the yeast assay system suggests a regulatory role of heterotrimeric G-protein subunits in dynein function. Indeed, both G\(\alpha\)s and G\(\beta\)\(\gamma\) are implicated in various aspects of membrane trafficking, cytoskeletal dynamics, and vesicular transport (18–22).

AGS3 consisted of 1500 nt and contained a truncated open reading frame at its 5’ terminus with a methionine embedded in a Kozak’s consensus sequence for translational initiation. The bioactivity/expression of this open reading frame (74 amino acids) was confirmed by mutational analysis and immunoblotting with specific peptide antiserum (data not shown). The AGS3 peptide was generated as a GST fusion protein and evaluated for specific interactions with purified G-protein subunits (Fig. 2B). AGS3 specifically bound recombinant G\(\alpha\)z, but it did not interact with purified brain G\(\beta\)\(\gamma\) (Fig. 2B). AGS3 preferred the G\(\beta\)\(\gamma\) subunit with a G204A mutant (Fig. 2B). AGS2 and AGS3 also did not alter the GDP-bound conformation of G\(\alpha\)z as described earlier for the AGS3 mutant (data not shown), which again contrasts with the effects of AGS1 and the NG108-15 G-protein activator (4, 5).2 These data suggest that although AGS2 and AGS3 clearly activate the pheromone response pathway at the level of G-protein, this event does not require the generation of GoGTP. By binding to G-protein subunits, AGS2 and AGS3 may inhibit heterotrimer formation or actively promote subunit dissociation “releasing” G\(\beta\)\(\gamma\). Such mechanisms of G-protein activation dramatically differ from that involving a typical G-protein-coupled receptor and might lead to selective activation of G\(\beta\)\(\gamma\)-regulated effectors. Alternatively, AGS2 and AGS3 may position G-proteins within a signal transduction complex or regulate signal processing by compartmentalization. Increasing evidence indicates that G\(\alpha\)s and G\(\beta\)\(\gamma\) may exist independently of each other in the cell, and perhaps AGS2 and AGS3 serve as alternative functional binding partners for the G-protein subunits (20–22).

A full-length rat AGS3 cDNA (650 amino acids; AF107723) was identified by screening a rat brain cDNA library and subsequent 5’-RACE (Fig. 3A). The full-length AGS3 protein, generated as an amino-terminal His-tagged protein in Sf9 cells, also interacts with GoGDP as described earlier for the AGS3 subdomain isolated in the yeast screen. Endogenous full-length AGS3 and endogenous G\(\alpha\)z apparently form complexes within the cell as determined in co-immunoprecipitation experiments.

3 M. J. Cismowski, A. Takesono, S. M. Lanier, and E. Duzic, unpublished observations.
with crude cell lysates. Blastp analysis of nonredundant GenBank CDS translations, PDB, SwissProt, and PIR indicated that full-length AGS3 exhibited homology with the partial mouse cDNA l23316 (96% identity, 97% similarity), the human LGN protein (59% identity, 66% similarity) and a predicted Caenorhabditis elegans protein (PID-g1065449; gene F32A6.4, U40409) (30% identity, 42% similarity). LGN was isolated as a truncated carboxyl-terminal fragment in a two-hybrid screen using Gαα as “bait” (24). Analysis of human and mouse EST data bases indicated that AGS3 and LGN are not species homologs and likely encode two distinct members of a larger protein family defined by conserved structural motifs and functional properties. AGS3, LGN, and the predicted C. elegans protein actually possess two defined structural cassettes that essentially divide the protein in half. The amino-terminal half of the AGS3 consists of six tetratricopeptide repeats, which serve as protein interaction motifs and regulatory domains in various proteins. The tetratricopeptide repeats in AGS3 may function as a regulatory domain controlling the bioactivity of the carboxyl-terminal region or the trafficking of AGS3 and associated proteins within the cell.

The carboxyl-terminal half of AGS3 and the two related proteins contains four repeated sequences of 18–19 amino acids that exhibit 80–85% homology. One or two such repeat domains are also found in four proteins that influence the nucleotide-binding properties or GTPase activity of G-proteins (Fig. 3B). Ppcl7 (PQ0109) (Ppc2) was isolated in a yeast two-hybrid screen using Gαα as bait and may act as a guanine nucleotide exchange factor (25). RAP1GAP (P47736) is a GTPase-activating protein for the small G-protein RAP-1A and was also isolated in a two-hybrid screen using “activated” Gαα as bait (26). RGS12 (AF035151) and RGS14 (O08773) are members of the family of “regulators of G-protein signaling.”

Further analysis indicated that each GPR motif may exist as an amphipathic helix. The AGS3 cDNA isolated in the yeast screen actually contained one complete GPR motif. Introduction of mutations into this GPR motif that disrupt the predicted amphipathic helix eliminated interaction of AGS3 with Gαα and Gαααα in crude cell extracts (Fig. 3B), and the same fusion proteins were inactive in the yeast assay system (data not shown). As observed for the interaction between AGS3 and purified recombinant Gαααα (Fig. 2B), AGS3 preferentially interacted with GoGDP versus GTPγS in the crude cell lysate (Fig. 3C). Despite the presence of GDP, which would stabilize the G-protein heterotrimer, the AGS3-GoGDP complex from the mammalian cell lysate did not contain Gβγ. In contrast, Gβγ subunits were readily detected when GoGDP was isolated from the same cell extract by immunoprecipitation with a Go antibody (Fig. 3C). These data support the hypothesis that AGS3 activates G-protein signaling by influencing subunit interactions. Alternatively, it is possible that AGS3 is selectively interacting with a population of Go in the cell that exists independent of Gβγ and subserves unexpected functional roles.

AGS proteins are indicative of a growing number of accessory proteins that influence signal propagation by heterotrimeric G-protein systems (3, 4, 19, 25, 26). Such entities may influence the population of activated G-protein within the cell independent of external stimuli or provide a cell-specific mechanism for signal amplification by acting in concert with G-protein-coupled receptors. Such proteins also provide a mechanism for signal input to heterotrimeric G-protein signaling systems that is distinct from that initiated by a seven-membrane span hormone receptor. By virtue of their distinct properties, AGS2 and AGS3 may also belong to a larger group of proteins that serve as binding partners for Ga and Gbg allowing the subunits to subserve functions that do not require initial heterotrimer formation.

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REFERENCES

1. Wu, G., Krupnick, J. G., Benovic, J. L., and Lanier, S. M. (1997) J. Biol. Chem. 272, 17836–17842
2. Wu, G., Hildebrandt, J., Benovic, J. L., and Lanier, S. M. (1998) J. Biol. Chem. 273, 7197–7200
3. Sato, M., Kataoka, R., Dingus, J., Wilcox, M., Hildebrandt, J., and, Lanier, S. M. (1995) J. Biol. Chem. 270, 15269–15276
4. Sato, M., Ribas, C., Hildebrandt, J. D., and Lanier, S. M. (1996) J. Biol. Chem. 271, 30052–30060
5. Cisowski, M., Takesono, A., Ma, C., Lizano, J. S., Xie, S., Fuernkranz, H., Lanier, S. M., and Duzic, E. (1999) Nature Biotechnol. 17, 878–883
6. Schultz, J., Ferguson, B., and Sprague, G. F., Jr. (1995) Curr. Opin. Genet. Dev. 5, 31–37
7. Schultz, J., Milpitz, F., Bork, P., and Ponting, C. P. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5857–5864
8. Bailey, T. L., and Gribskov, M. J. (1997) J. Comput. Biol. 4, 45–59
9. Fischer, D., and Eisenberg, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11929–11934
10. Holbrook, S. R., Dubekal, I., and Kim, S. H. (1993) BioTechniques 14, 894–899
11. Klein, C., Paul, J. I., Saure, K., Schmidt, M. M., Arangoli, L., Ransom, J., Trueheart, J., Manfredi, J. P., Bocko, R., and Murphy, A. J. (1998) Nature Biotechnol. 16, 1334–1337
12. King, S. M., Dillman, J. F., III, Benasheki, S. E., Lye, R. J., Patel-King, R. S., and Buder, A. (1998) Cell 97, 877–887
13. Lagriffoul, A., Olds-Clarke, P., and King, S. M. (1998) J. Cell Biol. 140, 1137–1147
14. Tai, A. W., Chuang, J. Z., and Sung, C. H. (1998) J. Biol. Chem. 273, 19639–19649
15. Nagano, F., Orita, S., Sasaki, T., Naito, A., Sakaguchi, G., Maeda, M., Watanabe, T., Kominami, E., Uchiyama, Y., and Takai, Y. (1998) J. Biol. Chem. 273, 30065–30068
16. Campbell, K. S., Cooper, S., Dessing, M., Yates, S., and Buder, A. (1998) J. Immunol. 161, 1728–1737
17. Tai, A. W., Chuang, J. Z., Bode, C., Wolfkum, U., and Sung, C. H. (1997) Cell 90, 59–68
18. Lagriffoul, A., Charpentier, N., Carrette, J., Tougard, C., Bockaert, J., and Homburger, V. (1996) J. Biol. Chem. 271, 32261–32267
19. Harrison, A., Olds-Clarke, P., and King, S. M. (1998) J. Cell Biol. 140, 1137–1147
20. Tai, A. W., Chuang, J. Z., and Sung, C. H. (1998) J. Biol. Chem. 273, 19639–19649
21. Denker, S. P., Mccaffery, J. M., Palade, G. E., Insel, P. A., and Farquhar, M. G. (1996) J. Cell Biol. 133, 1027–1040
22. Amador, C., Yamamoto, N., Van Lint, J., Ladenslagler, J., Van den Heede, J. R., Faulkner, D. J., and Malhotra, V. (1999) Cell 96, 59–68
23. Watson, N., Linder, M., Drucey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) Nature 383, 172–175
24. Mochizuki, N., Cho, G., Wen, B., and Insel, P. A. (1996) Gene (Amst.) 181, 39–43
25. Luo, Y., and Denker, B. M. (1999) J. Biol. Chem. 274, 10685–10688
26. Jordan, J. D., Carey, K. D., Stork, P. J. S., and Iyengar, R. (1999) J. Biol. Chem. 274, 21507–21510

M. Bernard and S. M. Lanier, unpublished observations.