AD_______________

Award Number: W81XWH-04-1-0208

TITLE: Novel Gbeta Mimic Kelch Proteins Gpb1 and Gpb2 Connect G-Protein Signaling to Ras Via Yeast Neurofibromin Homologs Ira 1 and Ira 2: A Model for Human NF1

PRINCIPAL INVESTIGATOR: Joseph Heitman, M.D., Ph.D.
Toshiaki Harashima, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

REPORT DATE: March 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20060503138

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2005	Annual (1 Mar 2004 - 28 Feb 2005)

4. TITLE AND SUBTITLE
Novel Gbeta Mimic Kelch Proteins Gpb1 and Gpb2 Connect G-Protein Signaling to Ras Via Yeast Neurofibromin Homologs Ira 1 and Ira 2: A Model for Human NF1

5. FUNDING NUMBERS
W81XWH-04-1-0208

6. AUTHOR(S)
Joseph Heitman, M.D., Ph.D.
Toshiaki Harashima, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: Heitm001@duke.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The Neurofibromatosis type 1 (*NF1*) gene encodes a large tumor suppressor protein, neurofibromin, which is a Ras GTPase-activating protein (RasGAP) activity. Although the *NF1* gene was identified over a decade ago, the biological roles of neurofibromin in cellular processes remain unclear. Therefore it is crucial for therapy and developing new drugs for NF1 patients to elucidate how the RasGAP activity of neurofibromin is controlled. To achieve this goal, it is also important to identify regulatory elements for neurofibromin. We are investigating the molecular mechanisms by which the Ras GAP activity of the yeast neurofibromin homologs Ira1/2 is regulated as a model to understand human NF1. We have found that the kelch Gβ subunit mimics Gpb1/2 interact with Ira1/2 and control the Ras GAP activity of Ira1/2. Here, we found that the Gpb1/2 proteins are localized to the cell membrane in a Gpa2 dependent manner and function at the cell membrane. Gpb1/2 bind to the C-terminus of Ira1/2 and stabilize the Ira1/2 proteins. Moreover we also identified a Gpb1/2 binding domain near the C-terminus of Ira1/2 (GBD) that is significantly conserved in neurofibromin homologs, including a human counterpart. Therefore, similar regulatory mechanisms might be conserved in evolution.

14. SUBJECT TERMS	15. NUMBER OF PAGES
GTPase activating protein (GAP), Ras, yeast, signal transduction, adenylyl cyclase, protein kinase A	43
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover..p1

SF 298..p2

Introduction..p4

Body..p5

Key Research Accomplishments..p8

Reportable Outcomes..p9

Conclusions..p10

References..p11

Appendices..p15

Introduction

Neurofibromatosis type 1 (NF1) is one of the most common genetic disorders in humans and the Ras GTPase-activating protein (RasGAP), neurofibromin, is intimately associated with NF1 (For reviews, see Dasgupta and Gutmann, 2003; Parada, 2000; Zhu and Parada, 2002). Therefore it is important to elucidate molecular mechanisms governing the RasGAP activity of neurofibromin as well as the biological roles of neurofibromin, which are as yet incompletely understood. We employ the budding yeast *Saccharomyces cerevisiae* as a model to understand how the GAP activity of the yeast neurofibromin homologs, Ira1 and Ira2, is controlled. The biochemical and biological roles of these yeast homologs have been well conserved in evolution (Ballester et al., 1990; Ballester et al., 1989; Buchberg et al., 1990; Martin et al., 1990; Tanaka et al., 1991; Tanaka et al., 1989; Tanaka et al., 1990a; Tanaka et al., 1990b; Xu et al., 1990a; Xu et al., 1990b). Recently, we identified the kelch Gb mimic proteins Gpb1 and Gpb2, which are structurally and functionally related to Gb subunits yet share no primary sequence identity with known Gb subunits (Harashima and Heitman, 2002). We have found that Gpb1/2 physically bind to Ira1/2 in vivo and regulate the cAMP signaling pathway by inhibiting the Ga subunit Gpa2 and by concomitantly stabilizing and thereby activating the Ira1/2 RasGAPs. In the approved Statement Of Work (See appendices), we proposed to elucidate the roles of the Gb mimic kelch proteins Gpb1/2 in regulating the yeast neurofibromin homologs Ira1/2 for the first year of this project. Here, we report that Gpb1/2 are localized to the cell membrane in a Gpa2-dependent manner and function at the cell membrane where Gpb1/2 bind to the C-terminus of Ira1/2 and stabilize the Ira1/2 proteins.

Body

A carboxy terminal domain of Ira1 spanning from amino acids 2715 to 2925 was identified as the Gpb1/2 binding domain (GBD). To understand how Gpb1/2 control Ira1/2 RasGAP activity, the Gpb1/2 binding domain on Ira1/2 was identified. In this study, the Ira1 protein was deleted for N-terminal and C-terminal regions and fused to the 3HA protein tag. Using these deletion constructs and FLAG-Gpb1/2 constructs, physical protein interactions were examined in vivo by FLAG tag based affinity purification methods, and a Gpb1/2 binding domain (GBD) was identified and mapped to a carboxy-terminal segment spanning amino acid residues 2715-2925 (Figure 1 in Supporting Data). The GBD in the Ira2 protein was also identified in the corresponding region of Ira2 (Figure 2 in Supporting Data).

The GBD is significantly conserved in evolution. To examine whether the GBD is conserved in evolution, we performed psi-BLAST searches in the NCBI database using the amino acid sequence of the GBD from Ira1. This search reveals identity with neurofibromin homologs including one in Drosophila, mouse, and human. Therefore, the GBD is conserved in evolution. Importantly many mutations (including nonsense mutations, deletions, and mutations in splice sites) have been identified in the corresponding domain of human neurofibromin from NF1 patients (Ars et al., 2003; De Luca et al., 2003; Fahsold et al., 2000; Origone et al., 2002; Rasmussen and Friedman, 2000; Upadhyaya et al., 1997).

Binding of Gpb1/2 to the GBD stabilizes the Ira1/2 proteins. In parallel with the experiments described above, protein stability of the deletion derivatives was also assessed by western blot using anti-HA antibodies. Remarkably, the deletion of the C-terminus resulted in instability of Ira1/2 (Figures 1 and 2 in Supporting Data). Furthermore, the protein levels of Ira1/2 were dramatically reduced in *gpb1,2* double mutant cells compared to wild-type cells (Figure 3 in Supporting Data). RT-PCR analysis of *IRA1*/2 expression revealed comparable transcript levels between *gpb1,2* mutant and wild-type cells. Reintroduction of the *GPB1/2* genes into *gpb1,2* double mutant cells restored Ira1/2 protein levels to the wild-type level (Figure 3 in Supporting Data). Therefore, Gpb1/2 stabilize the Ira1/2 proteins by binding to the GBD.

Gpa2 is required for membrane localization of Gpb1/2. Ras and RasGAP Ira1/2 are associated with the cell membrane. When a functional GFP tagged Gpb2 (GFP-Gpb2) was expressed without Gpa2, the GFP-Gpb2 protein was observed in the cytoplasm (Figure 4 in Supporting Data). However when the GFP-Gpb2 protein was coexpressed with the wild-type Gpa2 protein that is tethered to the cell membrane, or with a nuclear localization signal containing Gpa2 protein that is predominantly localized to the nucleus, it was recruited to the cell membrane or to the nucleus, respectively (Figure 4 in Supporting Data). The Gpa2^{G2A} protein that is no longer associated with the cell membrane could not direct GFP-Gpb2 to the cell membrane (Figure 4 in Supporting Data). Therefore Gpb2 is recruited to the cell membrane in a Gpa2-dependent fashion (see Appendices and Harashima and Heitman, 2005).

Membrane associated Gpb1/2 proteins are functional. We found that Gpb1/2 were recruited to the cell membrane by Gpa2, and the first ten amino acids ($Gpa2^{1-10}$) from Gpa2 are sufficient for membrane localization (Figure 5 in Supporting Data). To examine if membrane localization is required for Gpb1/2 function, Gpb1/2 were fused to a $Gpa2^{1-10}$-GFP construct that directs localization to the cell membrane and then expressed in *gpb1,2* double mutant cells. Gpb1/2 were also fused to a nuclear localization signal containing GFP construct (NLS-GFP) to promote localization to the nucleus. We found that the $Gpa2^{1-10}$-GFP-Gpb1/2 protein was efficiently localized to the cell membrane and able to complement the enhanced pseudohyphal phenotype of *gpb1,2* double mutant cells (Figure 6 in Supporting Data). On the other hand, the NLS-GFP-Gpb1/2 protein was largely localized to the nucleus and unable to complement the *gpb1,2* double mutant phenotype (Figure 6 in Supporting Data). Therefore, the Gpb1/2 proteins function on the cell membrane (see Appendices and Harashima and Heitman, 2005).

Key Research accomplishments

1. The kelch Gβ mimic Gpb1/2 proteins are recruited to the plasma membrane in a Gα Gpa2-dependent manner.
2. Gpb1/2 function at the cell membrane.
3. The amino acids 2715-2925 in Ira1 and the corresponding region in Ira2 were identified as the GBD (Gpb1/2 binding domain) that is conserved in evolution.
4. Gpb1/2 stabilize the yeast neurofibromin homologs Ira1/2 by binding to the GBD.

Reportable outcomes

1. A senior research associate in my laboratory who is working on this project received the Young Investigator Award from the Children's Tumor Foundation (formerly the National Neurofibromatosis Foundation) in 2004.
2. Our study entitled "Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP induced dimorphic transitions in *Saccharomyces cerevisiae*" was published in *Molecular Biology of the Cell* in 2005 (See Appendices).

Conclusions

In this period of time, we identified the Gpb1/2 binding domain (GBD) near the C-terminus of the neurofibromin homologs Ira1/2. Furthermore we found that Gpb1/2 stabilize Ira1/2 by binding to the GBD. Therefore loss of Gpb1/2 results in a decrease of the RasGAP Ira1/2 proteins and consequently to an increase in the GTP bound form of Ras, which is the active form of Ras and ultimately associated with NF1. Importantly the GBD is significantly conserved in neurofibromin homologs, including the human counterpart, and mutations that lead to loss of the GBD have been identified from NF1 patients. Therefore the same regulatory mechanisms may be conserved in evolution, and this study could provide information as to how the RasGAP activity of neurofibromin is regulated and ultimately provide therapeutic clues for NF1 patients and possible avenues for novel drug development.

References

Ars, E., Kruyer, H., Morell, M., Pros, E., Serra, E., Ravella, A., Estivill, X., and Lázaro, C. (2003). Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. J Med Genet *40*, e82.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990). The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. Cell *63*, 851-859.

Ballester, R., Michaeli, T., Ferguson, K., Xu, H. P., McCormick, F., and Wigler, M. (1989). Genetic analysis of mammalian GAP expressed in yeast. Cell *59*, 681-686.

Buchberg, A. M., Cleveland, L. S., Jenkins, N. A., and Copeland, N. G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and *IRA-1* and *IRA-2* negative regulators of the *RAS* cyclic AMP pathway. Nature *347*, 291-294.

Dasgupta, B., and Gutmann, D. H. (2003). Neurofibromatosis 1: closing the GAP between mice and men. Curr Opin Genet Dev *13*, 20-27.

De Luca, A., Buccino, A., Gianni, D., Mangino, M., Giustini, S., Richetta, A., Divona, L., Calvieri, S., Mingarelli, R., and Dallapiccola, B. (2003). NF1 gene analysis based on DHPLC. Hum Mutat *21*, 171-172.

Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kücükceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., *et al.* (2000). Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am J Hum Genet *66*, 790-818.

Harashima, T., and Heitman, J. (2002). The Gα protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gβ subunits. Mol Cell *10*, 163-173.

Harashima, T., and Heitman, J. (2005). Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP induced dimorphic transitions in *Saccharomyces cerevisiae*. Mol Biol Cell, published online July 19, 2005 as 10.1091/mbc.E05-04-0330.

Martin, G. A., Viskochil, D., Bollag, G., McCabe, P. C., Crosier, W. J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R. M., and et al. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell *63*, 843-849.

Origone, P., De Luca, A., Bellini, C., Buccino, A., Mingarelli, R., Costabel, S., La Rosa, C., Garrè, C., Coviello, D. A., Ajmar, F., *et al.* (2002). Ten novel mutations in the human neurofibromatosis type 1 (NF1) gene in Italian patients. Hum Mutat *20*, 74-75.

Parada, L. F. (2000). Neurofibromatosis type 1. Biochim Biophys Acta *1471*, M13-19.

Rasmussen, S. A., and Friedman, J. M. (2000). *NF1* gene and neurofibromatosis 1. Am J Epidemiol *151*, 33-40.

Tanaka, K., Lin, B. K., Wood, D. R., and Tamanoi, F. (1991). *IRA2*, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proc Natl Acad Sci U S A *88*, 468-472.

Tanaka, K., Matsumoto, K., and Toh-e, A. (1989). *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol Cell Biol *9*, 757-768.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990a). *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. Cell *60*, 803-807.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990b). *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian *ras* GTPase-activating protein. Mol Cell Biol *10*, 4303-4313.

Upadhyaya, M., Osborn, M. J., Maynard, J., Kim, M. R., Tamanoi, F., and Cooper, D. N. (1997). Mutational and functional analysis of the neurofibromatosis type 1 (*NF1*) gene. Hum Genet *99*, 88-92.

Xu, G. F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990a). The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. Cell *63*, 835-841.

Xu, G. F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., and et al. (1990b). The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell *62*, 599-608.

Zhu, Y., and Parada, L. F. (2002). The molecular and genetic basis of neurological tumours. Nat Rev Cancer *2*, 616-626.

Appendices

Statement of Work

Task 1. To characterize the roles of the Gβ mimic kelch proteins Gpb1 and Gpb2 in regulating the yeast neurofibromin homologs Ira1 and Ira2 (Months 1-12):

a. Determine the role of Gpb1/2 on Ira1/2 (Months 1-4.5)
    I. Construct and develop materials required for GAP assay of Ira1/2 (Months 1-3).
    II. Perform GAP assay to examine the roles of Gpb1/2 on Ira1/2 RasGAP activity (Months 3-4.5).
b. Identify the Gpb1/2-binding domain on Ira1/2 (Months 1-6):
    I. Construct Ira1/2 derivatives carrying various deletions in the N-terminal, central, and C-terminal regions (Months 1-4.5).
    II. Test protein-protein interactions and identify the Gpb1/2-binding domain (Months 4.5-6).
c. Identify amino acid residues in Ira1/2 required for protein-protein interactions with Gpb1/2 (Months 6-12):
    I. Mutagenize the Gpb1/2-interacting domain in Ira1/2 and clone into the yeast two-hybrid vector (Months 6-7).
    II. Test protein-protein interactions and identify amino acids required for physical interactions with Gpb1/2 (Months 7-9).

III. Introduce mutations in the *IRA1/2* genes that abolish physical interactions with Gpb1/2 in vivo (Months 9-11).

IV. Test for pseudohyphal differentiation to characterize the role of the mutated amino acids in vivo (Months 11-12).

Task 2. To identify amino acid residues important for function of neurofibromin and Ira1/2 (Months 12-24):

a. Construct and express the *NF1* gene in yeast *ira1,2* mutants to examine whether the full length neurofibromin is functional when heterologously expressed in yeast cells (Months 12-13).

b. To identify putative Gpb1/2 binding sites in neurofibromin (Months 13-24):

I. Introduce mutations in those ones of neurofibromin and clone these novel *NF1* alleles into yeast and mammalian expression vectors (Months 13-17).

II. Express these *NF1* alleles in the yeast *ira1,2* mutant and mouse $NF1^{-/-}$ cells and characterize the roles of the mutated amino acids in vivo (Months 17-24).

c. To characterize the roles of the consensus PKA phosphorylation sites in neurofibromin and Ira1/2 (Months 13-24):

I. Introduce mutations in candidate PKA phosphorylation sites in neurofibromin and Ira1/2 (Months 13-17).

II. Express these *NF1* mutant alleles in the yeast *ira1,2* mutant and mouse $NF1^{-/-}$ cells and the *IRA1/2* mutant alleles in the *ira1,2* mutant cells and test for phenotypes to examine the roles of those putative PKA phosphorylation sites (Months 17-24).

Task 3. To identify a human Gpb1/2 counterpart (Months 24-36):

a. To examine whether yeast Gpb1/2 interact with neurofibromin (Months 24-27):

   I. Construct FLAG-Gpb1/2 to be expressed and transfected into murine cells (Months 24-25).

   II. Examine protein-protein interactions by FLAG tag based immunopurification methods and western blots using anti-neurofibromin and anti-FLAG antibodies (Months 25-27).

b. To isolate a human Gpb1/2 counterpart (Months 27-36):

   I. Perform psi-BLAST searches against human sequence databases (Month 27).

   II. Make constructs for analysis in the yeast two-hybrid system and test protein-protein interactions between neurofibromin and putative Gpb1/2 counterparts (Months 27-31).

   III. Also generate yeast two-hybrid constructs of the candidate Gpb1/2 binding domain in neurofibromin and screen human

two-hybrid libraries to identify putative Gpb1/2 counterparts

(Months 31-36).

Supporting Data

Figure legends

Figure 1. Kelch Gpb1/2 subunits bind to the C-terminus of Ira1. The FLAG-Gpb1 (pTH111) and FLAG-Gpb2 (pTH88) fusion proteins were expressed in yeast cells that also express the 3HA tagged wild type Ira1 or Ira1 deletion variants and protein complexes were immunoprecipitated. (A) Schematic of Ira1 deletion proteins created and summary of results obtained from assays of protein stability and Gpb1/2 binding as below. Positions of deletions created in Ira1 are shown and numbered. A conserved region between Ira1/2 and the human neurofibromin protein is shaded in grey. The RasGAP related domain (GRD) and the Gpb1/2 binding domain (GBD) are shown as a hatched and lighter grey rectangle, respectively. (B) Protein interactions were investigated using crude cell extracts from cells expressing the 3HA tagged full length Ira1 (1-3092, THY355a) or Ira1 deletion variants (1-2925 (THY424a), 1-2714 (THY402a), 1-2432 (THY401a), and 1-1257 (THY404a)). Positions of full length wild-type Ira1 (1~3092 aa) and deletion variants (1~2925 and 1~1257 aa) are indicated to the left of the panel. Positions at which molecular weight markers (250, 210, and 148 kD) migrate are indicated to the right of the panels. The deletion of 167 amino acids from the Ira1 C-terminus leads to reduced protein levels of Ira1 from 3- to 7 fold in comparison of the full length Ira1 protein level and the further deletion (378 amino acids) results in undetectable levels. Note that some smaller Ira1-3HA species were also detected via the C-terminal HA tag, indicating that these are proteolysis products lacking N-terminal regions. This further supports the assignment of the GBD to the C-terminal region of Ira1. (C) N-terminal deletion Ira1 variants (2432~3092 aa (THY438a) and 2715~3092 aa

(THY440a)) were tested for interaction with Gpb1/2. Positions of the full length and N-terminal deletion Ira1 variants (2432~3092 and 2715~3092 aa) are indicated to the left of the panel. Because the levels of these Ira1 N- and C-terminal deletion variant proteins were too low to detect by western blot, the full length and deletion Ira1 proteins were immunoprecipitated using anti-HA agarose beads, eluted, subject to western analysis, and examined for protein stability and indicated as "Input" in (B) and (C). Fpr1 in crude cell extracts served as loading controls and were also shown as "Input".

Figure 2. The GBD in Ira2 maps to the equivalent C-terminal region of Ira1. (A) The protein structure of the Ira1/2 proteins is depicted schematically. Positions of deletions created in Ira1/2 are shown and numbered. (B) The Gpb1/2 biding site (GBD) on Ira2 was also determined by assessing protein interactions between Gpb1/2 and C-terminal (1- 2702 aa (THY 457a) and 1-2922 aa (THY 456a)) and N-terminal (2703-3079 aa (THY 466a) in C) Ira2 deletion variants. The migration positions of full length wild-type Ira2 (3079 aa) and Ira2 deletion variants (2922 aa) are indicated to the left of the panel. Positions at which molecular weight markers (250, 210, and 148 kD) migrated are also indicated to the right of the panels in B. Details are essentially as described in the legend to Figure 1, unless otherwise specifically noted.

Figure 3. Kelch subunits Gpb1/2 stabilize RasGAP Ira1/2. The *GPB1* (pTH26) and *GPB2* (pTH114) genes were introduced into yeast strains THY427a (*IRA1-3HA*), THY428a *(IRA2-3HA*), THY425a (*gpb1,2 IRA1-3HA*), and THY426a (*gpb1,2 IRA2-3HA*) to examine protein stability of Ira1/2 and the interactions between Ras2 and Ira1/2.

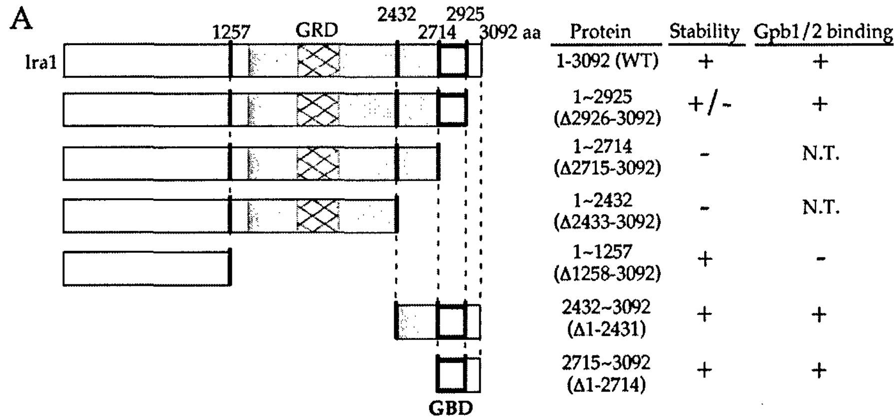
The Ira1/2-Ras2 protein complex was co-immunoprecipitated using anti-HA conjugated agarose gels and eluted by the addition of HA peptide (shown as "Co-IP (HA)" in upper panel). Fpr1 served as a loading control. "NT" indicates the non-tagged, wild-type Ira1 or Ira2 protein. Based on densitometric analysis the steady state protein levels of Ira1 and Ira2 were reduced in *gpb1,2* double mutant cells by at least 2- to 10-fold compared to wild-type cells. (Lower panel) Total RNA was prepared from cells as indicated and used for RT-PCR to test for expression of the *IRA1*, *IRA2*, and *ACT1* genes. Different cycles of PCR (20, 25, and 30 cycles) were employed to amplify products. Total RNA was also used for northern blots of the *ACT1* gene as a loading control.

Figure 4. Ga subunit Gpa2 recruits the kelch Gb subunit mimic Gpb2 to the plasma membrane. A functional GFP-Gpb2 protein (pTH84) was co-expressed with Gpa2 (pTH47), $Gpa2^{G299A}$ (pTH49), $Gpa2^{G2A}$ (pTH62), or $Gpa2^{G2A}$-NLS (pTH149) proteins in *gpa2*D mutant cells (MLY212a/a) and protein localization was investigated by direct fluorescence microscopy. The empty vector pTH19 ("-") served as control. Nuclear localization was confirmed by DAPI staining (data not shown). Scale bar, 5 μm.

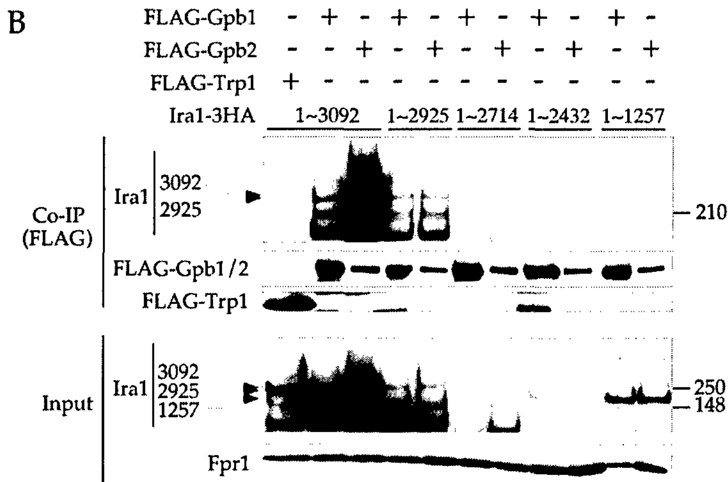
Figure 5. The first ten amino acids from Gpa2 are sufficient for membrane localization. A functionally, internally GFP tagged Gpa2 (Gpa2, pTH80), truncated GFP-tagged Gpa2 proteins, $Gpa2^{1-10}$-GFP ($Gpa2^{1-10}$, pTH71), $Gpa2^{1-20}$-GFP-FLAG ($Gpa2^{1-20}$, pTH81), and $Gpa2^{1-30}$-GFP ($Gpa2^{1-30}$, pTH65) were expressed from a 2m plasmid in wild-type yeast cells (MLY61a/a) to test for protein localization. The GFP cassette alone ("-", pTH73) was also expressed as a control. Scale bar, 5 μm.

Figure 6. Kelch Gβ mimic proteins Gpb1/2 function on the plasma membrane. A membrane localization sequence (MLS) or nuclear localization signal (NLS) was fused to the N-terminus of the functional GFP-Gpb1/2 proteins (pTH106/pTH75) and the resulting fusion proteins (pTH163, pTH164, pTH166, or pTH167) were expressed in diploid *gpb1,2* double mutant cells (THY212a/a) to test for protein localization (A) and function (B). The MLS-GFP-Gpb1/2 fusion proteins were recruited to the plasma membrane and were as functional as the wild-type Gpb1/2 proteins, whereas the NLS-GFP-Gpb1/2 fusion proteins were directed to the nucleus and nonfunctional. Cells bearing the empty vector (pTH19) or the *GPB1* (pTH26) or *GPB2* (pTH27) plasmid served as controls. Scale bar, 5 μm.

Figure 1.



Protein	Stability	Gpb1/2 binding
1-3092 (WT)	+	+
1~2925 (Δ2926-3092)	+/-	+
1~2714 (Δ2715-3092)	-	N.T.
1~2432 (Δ2433-3092)	-	N.T.
1~1257 (Δ1258-3092)	+	-
2432~3092 (Δ1-2431)	+	+
2715~3092 (Δ1-2714)	+	+



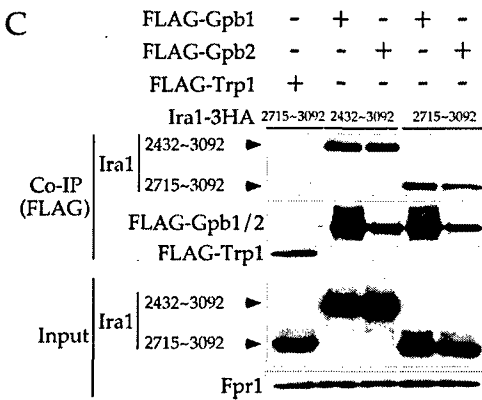


Figure 2.

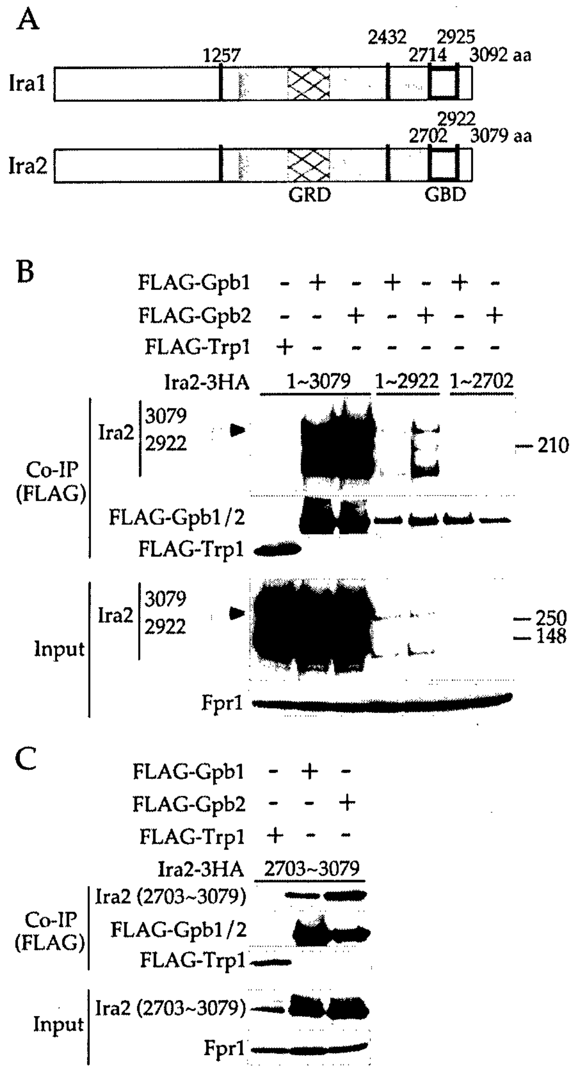


Figure 3.

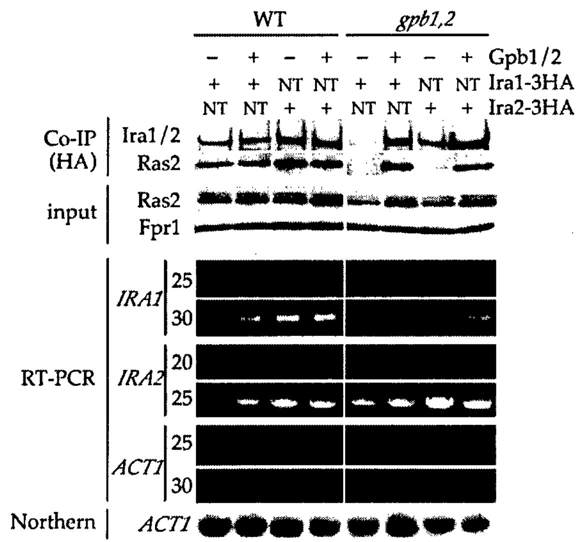


Figure 4.

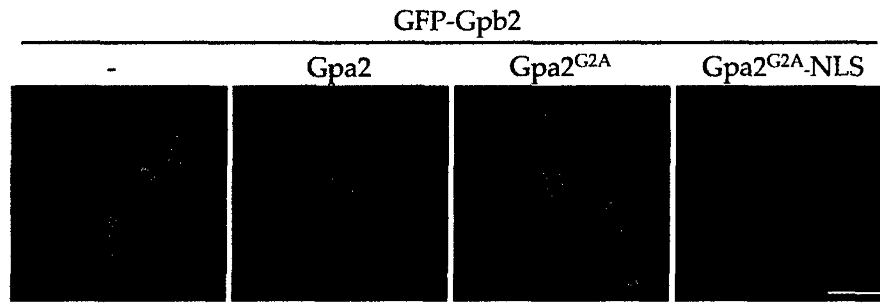


Figure 5.

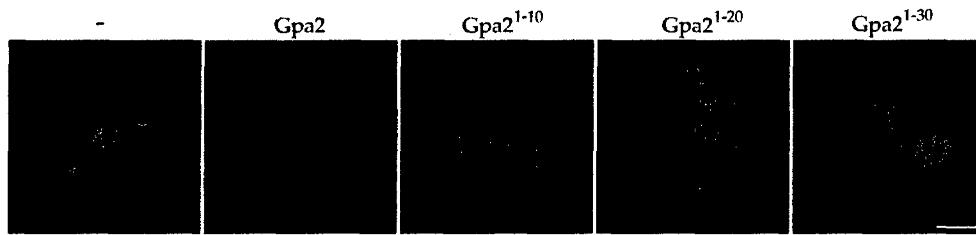
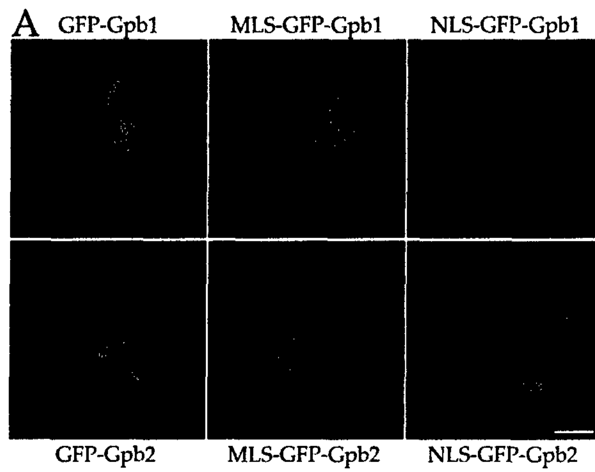
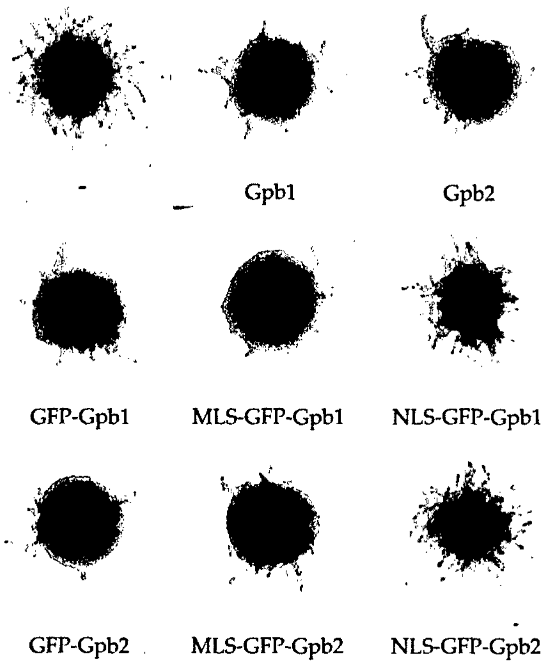


Figure 6.





Molecular Biology of the Cell
Vol. 16, 4557–4571, October 2005

Gα Subunit Gpa2 Recruits Kelch Repeat Subunits That Inhibit Receptor-G Protein Coupling during cAMP-induced Dimorphic Transitions in *Saccharomyces cerevisiae*

Toshiaki Harashima* and Joseph Heitman*†

*Department of Molecular Genetics and Microbiology and †Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710

Submitted May 9, 2005; Revised June 23, 2005; Accepted July 12, 2005
Monitoring Editor: Charles Boone

All eukaryotic cells sense extracellular stimuli and activate intracellular signaling cascades via G protein-coupled receptors (GPCR) and associated heterotrimeric G proteins. The *Saccharomyces cerevisiae* GPCR Gpr1 and associated Gα subunit Gpa2 sense extracellular carbon sources (including glucose) to govern filamentous growth. In contrast to conventional Gα subunits, Gpa2 forms an atypical G protein complex with the kelch repeat Gβ mimic proteins Gpb1 and Gpb2. Gpb1/2 negatively regulate cAMP signaling by inhibiting Gpa2 and an as yet unidentified target. Here we show that Gpa2 requires lipid modifications of its N-terminus for membrane localization but association with the Gpr1 receptor or Gpb1/2 subunits is dispensable for membrane targeting. Instead, Gpa2 promotes membrane localization of its associated Gβ mimic subunit Gpb2. We also show that the Gpa2 N-terminus binds both to Gpb2 and to the C-terminal tail of the Gpr1 receptor and that Gpb1/2 binding interferes with Gpr1 receptor coupling to Gpa2. Our studies invoke novel mechanisms involving GPCR-G protein modules that may be conserved in multicellular eukaryotes.

INTRODUCTION

All eukaryotic cells deploy on their surface signaling modules composed of G protein-coupled receptors (GPCR) and heterotrimeric G proteins to sense extracellular cues. GPCRs are conserved from yeasts to humans and constitute a family of cell surface receptors that contain seven transmembrane domains and sense myriad extracellular ligands including nutrients, odorants, hormones and pheromones, and photons (Gilman, 1987; Strader *et al.*, 1994; Lefkowitz, 2000; Mombaerts, 2004). Heterotrimeric G proteins consist of α, β, and γ subunits, in which the Gα subunits are guanine nucleotide binding proteins and the Gβγ subunits form a membrane-tethered heterodimer (Bourne, 1997; Sprang, 1997; Gautam *et al.*, 1998; Schwindinger and Robishaw, 2001; Cabrera-Vera *et al.*, 2003). Ligand binding triggers conformational changes in the GPCR that stimulate GDP-GTP exchange on Gα and release of the Gβγ dimer. Released Gα-GTP, Gβγ, or both signal downstream effectors. GTP-to-GDP hydrolysis (either intrinsic or RGS protein-stimulated) induces reassociation of the Gα-GDP subunit with Gβγ, extinguishing the signal (De Vries and Gist Farquhar, 1999; Guan and Han, 1999; Ross and Wilkie, 2000).

The yeast *Saccharomyces cerevisiae* expresses 3 GPCRs (Ste2, Ste3, and Gpr1) and 2 Gα subunits (Gpa1 and Gpa2), comprising two signaling modules: one that senses pheromones during mating and the other that senses nutrients and controls filamentous growth (Lengeler *et al.*, 2000; Harashima and Heitman, 2004). *S. cerevisiae* exists in two haploid mating types, **a** and α, which communicate via mating pheromones. **a** haploid cells express **a** pheromone and the GPCR Ste2 to sense extracellular α pheromone. α haploid cells express α pheromone and the GPCR Ste3 that senses **a** pheromone. In both cell types, Ste2 and Ste3 are coupled to the Gα subunit Gpa1, which forms a conventional heterotrimeric G protein with the Gβγ subunits Ste4/18. On pheromone binding to either receptor, GDP-GTP exchange occurs on Gpa1 and the Ste4/18 Gβγ complex dissociates. The liberated Ste4/18 dimer activates the pheromone responsive MAP kinase cascade culminating in mating (for reviews, see Dohlman and Thorner, 2001; Dohlman, 2002; Schwartz and Madhani, 2004).

In contrast to the pheromone GPCRs that are haploid- and mating-type-specific, a distinct GPCR, Gpr1, is expressed in both diploid and haploid cells. The Gpr1 receptor activates cAMP-PKA signaling and governs diploid pseudohyphal differentiation and haploid invasive growth via the coupled Gα subunit Gpa2 (for reviews, see Lengeler *et al.*, 2000; Pan *et al.*, 2000; Gancedo, 2001; Harashima and Heitman 2004). *gpr1* and *gpa2* mutants are defective in both pseudohyphal growth and transient cAMP production in response to glucose (Kübler *et al.*, 1997; Lorenz and Heitman, 1997; Colombo *et al.*, 1998; Yun *et al.*, 1998; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Rolland *et al.*, 2000; Tamaki *et al.*, 2000; Lemaire *et al.*, 2004). Recent studies provide evidence that glucose and structurally related sugars serve as ligands for the GPCR Gpr1 (Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Rolland *et al.*, 2000; Lemaire *et al.*, 2004).

The yeast Gα subunit Gpa2 shares 35–55% identity with other fungal and mammalian Gα subunits, and the predicted secondary structures are highly conserved between Gpa2 and canonical Gα subunits (Harashima and Heitman,

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-05-0403) on July 19, 2005.

Address correspondence to: Joseph Heitman (heitm001@duke.edu).

© 2005 by The American Society for Cell Biology

<zjs;Article> • <zjss;Articles> • <zdoi;10.1091/mbc.E05-05-0403>

2004). Amino acid residues that confer dominant phenotypes when mutated are also conserved. For instance, a mutation of Gln[300] to Leu (Q300L) in Gpa2 is analogous to the Giα1 Q204L mutation that abolishes the intrinsic GTPase activity and functions as an activated form of Gpa2 (Harashima and Heitman, 2002). A mutation of Gly[299] to Ala (Gpa2 G299A) is analogous to Giα1 G203A and Gαs G226A that fail to undergo the GTP-induced conformational change and thereby serves as a dominant negative allele and interacts with Gpb1/2 and Gpr1 more strongly compared with the wild-type Gpa2 (Lorenz and Heitman 1997; Harashima and Heitman, 2002).

Nevertheless, Gpa2 does not form a heterotrimeric complex with the known yeast G$\beta\gamma$ subunits Ste4/18 (Lorenz *et al.*, 2000; Harashima and Heitman, 2002, 2004). Recent studies identified two novel Gpa2 associated proteins, the kelch proteins Gpb1 and Gpb2, which are functionally redundant and share ~35% identity (Harashima and Heitman, 2002; Batlle *et al.*, 2003). The kelch motif is known to mediate protein-protein interactions (Adams *et al.*, 2000). Gpb1 and Gpb2 each contain seven kelch repeats, which share no sequence homology with the seven WD40 repeats of canonical Gβ subunits. The crystal structure of the kelch repeat enzyme galactose oxidase reveals that the seven kelch repeats can adopt a seven-bladed β-propeller structure strikingly similar to Gβ subunits (Ito *et al.*, 1991, 1994; Wall *et al.*, 1995; Lambright *et al.*, 1996; Sondek *et al.*, 1996; Adams *et al.*, 2000; Harashima and Heitman, 2002).

gpb1,2 mutants exhibit enhanced PKA phenotypes, including increased filamentous growth, sensitivity to nitrogen starvation and heat shock, reduced glycogen accumulation, and reduced sporulation (Harashima and Heitman, 2002; Batlle *et al.*, 2003). The *gpb1,2* mutant phenotypes are partially alleviated by *gpa2* mutations and abolished by mutation of the *TPK2* gene that encodes one of the three PKA catalytic subunits. These genetic findings support a model in which the kelch proteins Gpb1/2 negatively regulate the cAMP signaling pathway by inhibiting Gpa2 and an unidentified target that may be an upstream element of the PKA pathway including adenylyl cyclase or its regulator Ras or regulatory proteins of Ras (Harashima and Heitman, 2002).

In contrast to canonical Gα subunits, Gα Gpa2 has an extended N-terminus (Figure 1). This region shares no homology with known Gα subunits, whereas the remainder of Gpa2 shares >60% identity with Gα subunits in closely related yeasts and >40% identity with mammalian Gα subunits. The N-terminal regions of Gα subunits are known to mediate membrane localization and physical interactions with the cognate GPCR and G$\beta\gamma$ dimer (Navon and Fung, 1987; Hamm *et al.*, 1988; Journot *et al.*, 1991; Lambright *et al.*, 1996; Wall *et al.*, 1998; Yamaguchi *et al.*, 2003; Herrmann *et al.*, 2004).

All Gα subunits of heterotrimeric G proteins bear N-terminal lipid modifications (myristoylation and palmitoylation) necessary for membrane targeting (for reviews, see Chen and Manning, 2001; Cabrera-Vera *et al.*, 2003). Myristoylation involves the irreversible cotranslational addition of a 14-carbon myristoyl group on glycine at the second position in the consensus sequence MGXXXS and this occurs via an amide linkage after proteolytic removal of the initiating methionine (Johnson *et al.*, 1994; Ashrafi *et al.*, 1998; Farazi *et al.*, 2001). Palmitoylation occurs on all Gα subunits with the exception of Gαt (transducin) and involves posttranslational attachment of a saturated 16-carbon fatty acid, palmitate, via thioester linkage to cysteine residue(s) near the N-terminus. There is no palmitoylation consensus sequence, and palmitoylation is reversible and may be regulated. Both palmitoylation and myristoylation may play roles in addition to membrane localization (Linder *et al.*, 1991; Gallego *et al.*, 1992; Wedegaertner *et al.*, 1993; Wilson and Bourne, 1995; Wise *et al.*, 1997; Morales *et al.*, 1998; Evanko *et al.*, 2000; Fishburn *et al.*, 2000).

S. cerevisiae serves as a powerful model to study GPCR-G protein signaling (for reviews, see Jeansonne, 1994; Lengeler *et al.*, 2000; Dohlman and Thorner, 2001; Dohlman, 2002; Harashima and Heitman, 2004). The Gα subunit Gpa1 is myristoylated at the Gly[2] residue and palmitoylated at the Cys[3] residue (Song and Dohlman, 1996; Song *et al.*, 1996). Myristoylation is required for Gpa1 membrane targeting and palmitoylation, yet not for interaction with G$\beta\gamma$ (Song *et al.*, 1996). On the other hand, a Gpa1 palmitoylation-site mutant protein (Gpa1^{C3A}) is still partially localized to the plasma membrane, partially functional, and bound to G$\beta\gamma$ (Song and Dohlman, 1996). The G$\beta\gamma$ dimer, the associated GPCR Ste2/3, or components of the Gpa1 mediated MAP kinase cascade are not required for Gpa1 membrane localization (Song and Dohlman, 1996), but the Ste4/18 G$\beta\gamma$ dimer does promote receptor-Gpa1 coupling (Blumer and Thorner, 1990).

The distinct Gα subunit Gpa2 forms an unusual protein complex with the atypical binding partner kelch Gβ mimics Gpb1/2 and contains an extended N-terminus. Thus novel regulatory mechanisms may direct Gpa2 to the plasma membrane and enable Gpa2 to function as a molecular switch. Here we show that Gpa2 shares similar characteristics with Gpa1 involving lipid modifications and their function. Gpa2 interacting proteins are dispensable for Gpa2 membrane localization. However, unexpectedly, Gpa2 is required for membrane targeting of the kelch Gβ mimic Gpb2, in striking contrast to conventional heterotrimeric G proteins. Furthermore, the kelch Gβ mimic proteins Gpb1/2 were found to interfere with Gpr1 receptor-Gα Gpa2 coupling.

MATERIALS AND METHODS

Strains, Media, and Plasmids

Media and standard yeast experimental procedures were as described (Sherman, 1991). To express genes heterologously in yeast cells, an attenuated *ADH1* promoter and an *ADH1* terminator from the yeast two-hybrid vector pGBT9 were amplified by fusion PCR using primers, GCTTGCATGCAACT-TCTTTT/CGACGGATCCCCGGGAATTCCATCTTTCAGGAGGCTTGCT and AGCAAGCCTCCTGAAAGATGGAATTCCCGGGGATCCGTCG/CGGCATGCCGGTAGAGGTGT, for the 1st round PCR and primers, GCTT-GCATGCAACTTCTTTT/CGGCATGCCGGTAGAGGTGT for the second round PCR. The resulting PCR products were blunted with T4 DNA polymerase and cloned into the 2μ plasmid YEplac195 that was digested with *Hin*dIII and *Eco*RI and then blunted with T4 DNA polymerase to create a yeast expression vector pTH19 (*URA3* 2μ). pTH171 (*LEU2* 2μ), pTH172 (*TRP1* 2μ), and pTH173 (*LYS5* 2μ) are pTH19 derivatives. The nuclear localization signal (NLS) derived from the SV40 T antigen (PPKKKRKVA) was used to direct fusion proteins into the nucleus (Arévalo-Rodríguez and Heitman, 2005). pFA6a-GFP(S65T)-kanMX6 was used as the substrate for PCR to amplify GFP (Longtine *et al.*, 1998). Plasmids and yeast strains used in this study are listed in Tables 1 and 2. Details of plasmids and strains are available upon request.

AQ: A

T2

Pseudohyphal and Invasive Growth

Pseudohyphal and invasive growth assays were investigated as described previously (Harashima and Heitman, 2002).

Microscopic Studies

If not specifically described in figure legends, growth conditions were as follows. For protein localization study, cells were grown in synthetic minimal media to stationary phase and examined for protein localization under a fluorescent microscope (Zeiss Axioskop2 plus, Thornwood, NY) or a confocal microscope (Zeiss LSM 410).

Table 1. *S. cerevisiae* strains

Strain	Genotype	Source/Reference
Σ1278b congenic strains		
MLY40α	*MATα ura3-52*	Lorenz and Heitman (1997)
MLY61a/α	*MATa/α ura3-52/ura3-52*	Lorenz and Heitman (1997)
MLY97a/α	*MATa/α ura3-52/ura3-52 leu2Δ::hisG/leu2Δ::hisG*	Lorenz and Heitman (1997)
MLY132α	*MATα gpa2Δ::G418 ura3-52*	Lorenz and Heitman (1997)
MLY132a/α	*MATa/α gpa2Δ::G418/gpa2Δ::G418 ura3-52/ura3-52*	Lorenz and Heitman (1997)
MLY212a/α	*MATa/α gpa2Δ::G418/gpa2Δ::G418 ura3–52/ura3-52leu2Δ::hisG/leu2Δ::hisG*	Lorenz and Heitman (1997)
MLY232a/α	*MATa/α gpr1Δ::G418/gpr1Δ::G418 ura3–52/ura3-52*	Lorenz *et al.* (2000)
MLY277a/α	*MATa/α gpa2Δ::G418/gpa2Δ::G418 gpr1Δ::G418/gpr1Δ::G418 ura3-52/ura3-52*	Laboratory stock
THY212a/α	*MATa/α gpb1Δ::hph/gpb1Δ::hph gpb2Δ::G418/gpb2Δ::G418*	Harashima and Heitman (2002)
THY224a/α	*MATa/α gpg1Δ::hph/gpg1Δ::hph ura3–52/ura3-52*	This study
THY243a/α	*MATa/α gpb1Δ::hph/gpb1Δ::hph gpb2Δ::G418/gpb2Δ::G418 gpr1Δ::hph/gpr1Δ::hph ura3-52/ura3-52*	Harashima and Heitman (2002)
THY246a/α	*MATa/α gpb1Δ::hph/gpb1Δ::hph gpb2Δ::G418/gpb2Δ::G418 gpg1Δ::nat/gpg1Δ::nat ura3-52/ura3-52*	Harashima and Heitman (2002)
S288C background strains		
S1338	*MATa ura3Δ::loxP leu2Δ::loxP trp1Δ::loxP gal2*	Ito-Harashima
THY452	*MATa ura3Δ::loxP leu2Δ::loxP trp1Δ::loxP lys5Δ::loxP gal2*	This study

Preparation of Crude Cell Extracts and Immunoprecipitation

Total cell extracts from yeast cells that were grown to midlog phase ($OD_{600} \cong 0.8$) in synthetic dropout media were prepared in lysis buffer (50 mM HEPES, pH 7.6, 120 mM NaCl, 0.3% CHAPS, 1 mM EDTA, 20 mM NaF, 20 mM β-glycerophosphate, 0.1 mM Na-orthovanadate, 0.5 mM dithiothreitol, protease inhibitors (Calbiochem, La Jolla, CA; cocktail IV), and 0.5 mM phenylmethylsulfonyl fluoride) using a bead-beater. After centrifugation (25,000 × *g*, 20 min), crude extracts (2 mg) were mixed with anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) to precipitate FLAG tagged proteins.

In Vivo Lipid Modifications

Cells were grown in 10 ml of SD-Ura medium to $OD_{600} = 0.6–0.7$, collected, and resuspended into 5 ml of fresh SD-Ura medium. After 10 min, cerulenin was added at a final concentration of 2 μg/ml, and cells were incubated for an additional 15 min under the same conditions. Subsequently, [^{3}H]myristic acid or [^{3}H]palmitic acid was added to the cultures at a final concentration of 50 μCi/ml for myristoylation analysis or 500 μCi/ml for palmitoylation analysis. After 3 h, cells were collected and washed once with H_2O and twice with phosphate-buffered saline. Preparation of crude cell extracts and immunoprecipitation of FLAG tagged proteins were performed as above. The bound FLAG tagged proteins were eluted by boiling for 5 min in SDS-PAGE sample buffer in the presence of β-mercaptoethanol for the myristoylation analysis and in the absence of β-mercaptoethanol for the palmitoylation analysis (Song and Dohlman, 1996). After SDS-PAGE, gels were fixed in H_2O/2-propanol/acetic acid (65:25:10 vol/vol/vol) for 30 min and then soaked at room temperature for 18 h either in 1 M hydroxylamine (pH 7.0) to cleave thioester-linked fatty acids or 1 M Tris-HCl (pH 7.0) as a control. The gels were fixed again, treated with Amplify (Amersham, Piscataway, NJ) for 30 min, dried, and then exposed to an x-ray film (BioMax MS film, Eastman Kodak, Rochester, NY) with an intensifying screen (BioMax Transcreen LE, Kodak) at −80°C for 1–2 mo. Expression of the FLAG-tagged proteins was verified by Western blot analysis using anti-FLAG M2 antibody (Sigma).

cAMP Assay

cAMP assay was as described in Lorenz *et al.* (2000) with some modifications. Briefly, at the time points indicated, 0.5 ml of cell suspension was transferred into a microfuge tube containing 0.5 ml of 10% ice-cold trichloroacetic acid and was immediately frozen in liquid nitrogen. To prepare intracellular cAMP, cells were permeabilized by defrosting at 4°C overnight. Cell extracts were neutralized by ether extraction and lyophilized. Intracellular cAMP levels were determined by using a cAMP enzyme immunoassay kit (Amersham).

RESULTS

Gα Subunit Gpa2 Is Myristoylated and Palmitoylated

The Gα protein Gpa2 is coupled to the GPCR Gpr1 and signals to activate the downstream effector adenylyl cyclase in response to glucose. Based on analogy to other GPCR-Gα systems, we hypothesized that Gpa2 would be localized to the cell membrane for function. To address this, Gpa2 was fused to green fluorescent protein (GFP). To avoid perturbing protein localization or receptor coupling sequences typically linked to the amino and carboxy terminal regions of Gα proteins (Figure 1A), GFP was fused between the first 10 amino acids (1–10) of Gpa2 and the remainder of the protein (amino acids 4–449) to produce a $Gpa2^{1-10}$-GFP-$Gpa2^{4-449}$ internal fusion protein. This Gpa2-GFP fusion protein was functional based on its ability to complement the pseudohyphal defect of *gpa2* mutant cells (unpublished data). As shown in Figure 2A, the Gpa2-GFP fusion protein was localized to the cell membrane. A C-terminally GFP tagged Gpa2 protein was nonfunctional (unpublished data), in accord with the known role of the Gα C-terminal domain in receptor coupling (Slessareva *et al.*, 2003; Herrmann *et al.*, 2004).

F1 AQ:B F2

To establish the minimal Gpa2 domain required for membrane localization, the first 10 ($Gpa2^{1-10}$), 20 ($Gpa2^{1-20}$), or 30 ($Gpa2^{1-30}$) amino acids of Gpa2 were fused to a GFP cassette and expressed in vivo. All three C-terminally tagged Gpa2-GFP proteins were localized to the plasma membrane (Figure 2A). Therefore, as few as the first 10 amino acids of Gpa2 suffice for plasma membrane targeting.

In conventional Gα subunits, lipid modifications of the N-terminus mediate membrane localization (Chen and Manning, 2001). Myristoylation occurs at Gly^2 in the myristoylation consensus sequence G^2XXXS^6 (Johnson *et al.*, 1994). Palmitoylation can occur at any cysteine residue near the N-terminus. Gpa2 contains glycine and serine in the second and sixth positions for myristoylation and cysteine at the fourth position from the N-terminus. To examine whether these sites are lipid modified, a $Gpa2^{1-20}$-GFP-FLAG protein in which the first 20 amino acids of Gpa2 were fused to a GFP-FLAG cassette was expressed in yeast cells and assessed for lipid modifications. $Gpa2^{1-20}$-GFP-FLAG variants containing mutations in the potential lipid modification sites (G2A, C4A, or S6Y) were also analyzed.

As a positive control for lipid modification experiments, an equivalent $Gpa1^{1-20}$-GFP-FLAG protein was constructed, which was derived from the Gpa1 Gα subunit coupled to the

balt2/zmk-mbc/zmk-mbc/zmk01005/zmk7325-05a | eideg | S=9 | 9/2/05 | 9:29 | Art: | input-cip

Table 2. Plasmids

Plasmid	Description	Source/Reference
pTH19	P_{ADH1} *URA3* 2μ	This study
pTH26	P_{ADH1}-*GPB1 URA3* 2μ (pTH19)	This study
pTH27	P_{ADH1}-*GPB2 URA3* 2μ (pTH19)	This study
pTH47	P_{ADH1}-*GPA2 URA3* 2μ (pTH19)	This study
pTH48	P_{ADH1}-*GPA2*^{Q300L} *URA3* 2μ (pTH19)	This study
pTH49	P_{ADH1}-*GPA2*^{G299A} *URA3* 2μ (pTH19)	This study
pTH62	P_{ADH1}-*GPA2*^{G2A} *URA3* 2μ (pTH19)	This study
pTH65	P_{ADH1}-*GPA2*$^{1-30\ aa}$::*GFP URA3* 2μ (pTH19)	This study
pTH68	P_{ADH1}-*GPA2*^{C4A} *URA3* 2μ (pTH19)	This study
pTH69	P_{ADH1}-*GPA2*^{S6Y} *URA3* 2μ (pTH19)	This study
pTH71	P_{ADH1}-*GPA2*$^{1-10\ aa}$::*GFP URA3* 2μ (pTH19)	This study
pTH73	P_{ADH1}-*GFP URA3* 2μ (pTH19)	This study
pTH75	P_{ADH1}-*GFP-GPB2 URA3* 2μ (pTH19)	This study
pTH80	P_{ADH1}-*GPA2*$^{1-10}$::*GFP*::*GPA2*$^{4-449}$ *URA3* 2μ (pTH19)	This study
pTH81	P_{ADH1}-*GPA2*$^{1-20\ aa}$::*GFP-FLAG URA3* 2μ (pTH19)	This study
pTH84	P_{ADH1}-*GFP-GPB2 LEU2* 2μ (pTH171)	This study
pTH91	P_{ADH1}-*GPA2*$^{1-20\ aa\ G2A}$::*GFP-FLAG URA3* 2μ (pTH19)	This study
pTH92	P_{ADH1}-*GPA2*$^{1-20\ aa\ C4A}$::*GFP-FLAG URA3* 2μ (pTH19)	This study
pTH93	P_{ADH1}-*GPA2*$^{1-20\ aa\ S6Y}$::*GFP-FLAG URA3* 2μ (pTH19)	This study
pTH100	P_{ADH1}- *GFP-FLAG URA3* 2μ (pTH19)	This study
pTH103	P_{ADH1}-*GPA1*$^{1-20\ aa}$::*GFP-FLAG URA3* 2μ (pTH19)	This study
pTH106	P_{ADH1}-*GFP-GPB1 URA3* 2μ (pTH19)	This study
pTH114	P_{ADH1}-*GPB2 LEU2* 2μ (pTH171)	This study
pTH127	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-100}$ *URA3* 2μ (pTH19)	This study
pTH128	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-100\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH130	P_{ADH1}-*GPA2*$^{\Delta\alpha\ (51-57)\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH133	P_{ADH1}-*GPA2*$^{\Delta\alpha\ (51-57)}$ *URA3* 2μ (pTH19)	This study
pTH134	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-29\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH136	P_{ADH1}-*GPA2*$^{\Delta 16-84\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH144	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-14\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH145	P_{ADH1}-*GPA2*$^{\Delta 46-84\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH149	P_{ADH1}-*GPA2*^{G2A}-*NLS URA3* 2μ (pTH19)	This study
pTH155	P_{ADH1}-*GPA2*$^{\Delta 46-100}$ *URA3* 2μ (pTH19)	This study
pTH157	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-29}$ *URA3* 2μ (pTH19)	This study
pTH158	P_{ADH1}-*GPA2*$^{\Delta 46-84}$ *URA3* 2μ (pTH19)	This study
pTH159	P_{ADH1}-*GPA2*$^{\Delta 31-84\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH160	P_{ADH1}-*GPA2*$^{\Delta 31-84}$ *URA3* 2μ (pTH19)	This study
pTH161	P_{ADH1}-*GPA2*$^{\Delta 16-84}$ *URA3* 2μ (pTH19)	This study
pTH163	P_{ADH1}-*MLS-GFP-GPB2 URA3* 2μ (pTH19)	This study
pTH164	P_{ADH1}-*MLS-GFP-GPB1 URA3* 2μ (pTH19)	This study
pTH166	P_{ADH1}-*NLS-GFP-GPB2 URA3* 2μ (pTH19)	This study
pTH167	P_{ADH1}-*NLS-GFP-GPB1 URA3* 2μ (pTH19)	This study
pTH168	P_{ADH1}-*GPA2*$^{\Delta 46-100\ G299A}$ *URA3* 2μ (pTH19)	This study
pTH169	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-14}$ *URA3* 2μ (pTH19)	This study
pTH170	P_{ADH1}-*GFP-GPR1C TRP1* 2μ (pTH172)	This study
pTH171	P_{ADH1} *LEU2* 2μ	This study
pTH172	P_{ADH1} *TRP1* 2μ	This study
pTH173	P_{ADH1} *LYS5* 2μ	This study
pTH174	P_{ADH1}-*GPB1 LYS5* 2μ (pTH173)	This study
pTH178	P_{ADH1}-*GPA2*$^{\Delta 46-449}$ *URA3* 2μ (pTH19)	This study
pTH191	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-44}$ *URA3* 2μ (pTH19)	This study
pTH192	P_{ADH1}-*GPA1*$^{1-10}$-*GPA2*$^{\Delta 1-44\ G299A}$ *URA3* 2μ (pTH19)	This study

Ste2/3 pheromone receptors (Figure 2B). Gpa1 is known to be myristoylated at the second position on glycine (Gly^2) and palmitoylated on cysteine in the third position (Cys^3) (Song and Dohlman, 1996; Song *et al.*, 1996). Gpa1 myristoylation is essential for membrane localization and function and required for palmitoylation, and palmitoylation also promotes membrane localization and function. In addition, the first 9 amino acids of Gpa1 suffice for membrane localization of a Gpa1-GST fusion protein (Gillen *et al.*, 1998).

As shown in Figure 2B, the wild-type Gpa2 fusion protein was myristoylated and the myristoylation site and myristoylation consensus sequence mutant proteins, $Gpa2^{G2A}$ and $Gpa2^{S6Y}$, were not, suggesting that Gpa2 is subject to myristoylation at Gly^2. Gpa2 was also palmitoylated and a mutation in the putative palmitoylation site ($Gpa2^{C4A}$) abolished this modification (Figure 2C). Therefore, Gpa2 is also subject to palmitoylation at Cys^4. We note that the $Gpa2^{C4A}$ fusion protein exhibited a decreased level of myristoylation compared with the wild-type protein. Interestingly, reduced myristoylation was also observed with the $Gpa1^{C3S}$ mutant (Song and Dohlman, 1996). These results are indicative of either a sequence preference in the myristoylation consensus sequence (G^2XXXS^6) or a role for palmitoylation in promoting myristoylation or its maintenance.

Similar to Gpa1, Gpa2 requires myristoylation for palmitoylation because the G2A and S6Y mutations, which abolish

balt2/zmk-mbc/zmk-mbc/zmk01005/zmk7325-05a | eldeg | S=9 | 9/2/05 | 9:29 | Art | Input-cip

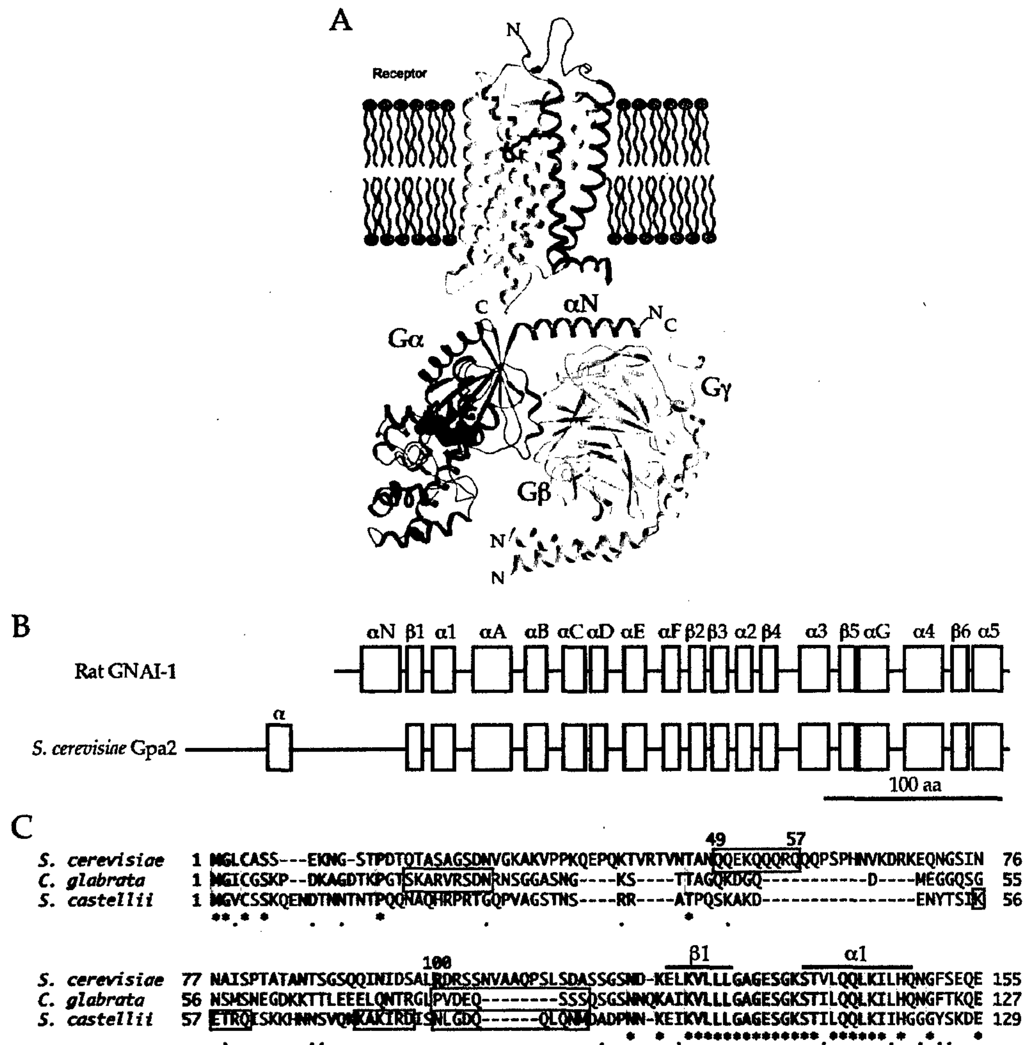


Figure 1. N-terminal alpha helix of Gα subunits (αN domain) is involved in receptor and Gβγ dimer coupling. (A) The αN domain provides one of the binding interfaces between Gα and Gβ and the receptor. This image shows a hypothetical model (PDB file 1BOK) for a GPCR-G protein module (GPCR; Rhodopsin, PDB file 1F88, G protein; PDB file 1GOT). The αN domain of the Gα subunit that is required for Gβ subunit and receptor coupling is shown (modified from Cabrera-Vera *et al.*, 2003). (B) The predicted secondary structures of the conventional rat Gαi subunit and the yeast Gα Gpa2 protein based on PHD (Rost *et al.*, 1993). Gpa2 shares 34% identity with the rat Gαi subunit and the predicted secondary structure is highly conserved between the two, except for the extended Gpa2 N-terminus. Secondary structure assignments were based on those of $G_{\alpha t/\alpha i}$ (Lambright *et al.*, 1996). (C) An alignment of the amino acid sequence of the N-terminus of Gpa2 homologues from *S. cerevisiae* and the related yeasts *C. glabrata* and *S. castellii*. *C. glabrata* and *S. castellii* express homologues of the *S. cerevisiae* GPCR Gpr1 and Gβ mimic Gpb1/2 proteins as well as a Gpa2 homologue, yet the N-termini of their Gpa2 homologues share no significant homology. Amino acids forming a potential alpha helix in the N-termini are indicated by red rectangles. Identical amino acids are marked (*) and shaded in gray, and conserved amino acids are also indicated (●). The 100th amino acid (R) of Gpa2 is shown in red. The β1 and α1 domains assigned in Figure 1B are shown. Alignments were obtained using Clustal W (Thompson *et al.*, 1994).

myristoylation, also blocked palmitoylation. Consistent with these results, the Gpa2-GFP-FLAG proteins bearing the G2A, C4A, or S6Y mutations failed to localize to the plasma membrane, and thus myristoylation and palmitoylation are required for Gpa2 plasma membrane localization (Figure 2A).

To address the physiological roles of these lipid modifications, the G2A, C4A, and S6Y mutations were introduced into the *GPA2* gene and expressed in a Σ1278b *gpa2/gpa2* diploid or *gpa2* haploid mutant strain. As shown in Figure 2, D and E, the $GPA2^{G2A}$ myristoylation site mutant failed to complement either the pseudohyphal or the invasive growth defects. The $GPA2^{S6Y}$ and $GPA2^{C4A}$ myristoylation consensus sequence or palmitoylation site mutants showed severe defects in both assays. Furthermore, introduction of a dominant active mutation (Q300L) that abolishes Gpa2 GTPase activity failed to restore activity of the $GPA2^{G2A}$ mutant protein ($Gpa2^{G2A,\ Q300L}$, unpublished data). Thus, myristoylation and palmitoylation both play critical roles in Gpa2 membrane localization and signaling. Importantly, the unusual Gα subunit Gpa2 shares common features with the conventional Gα subunit Gpa1 with respect to lipid modifications and their physiological roles.

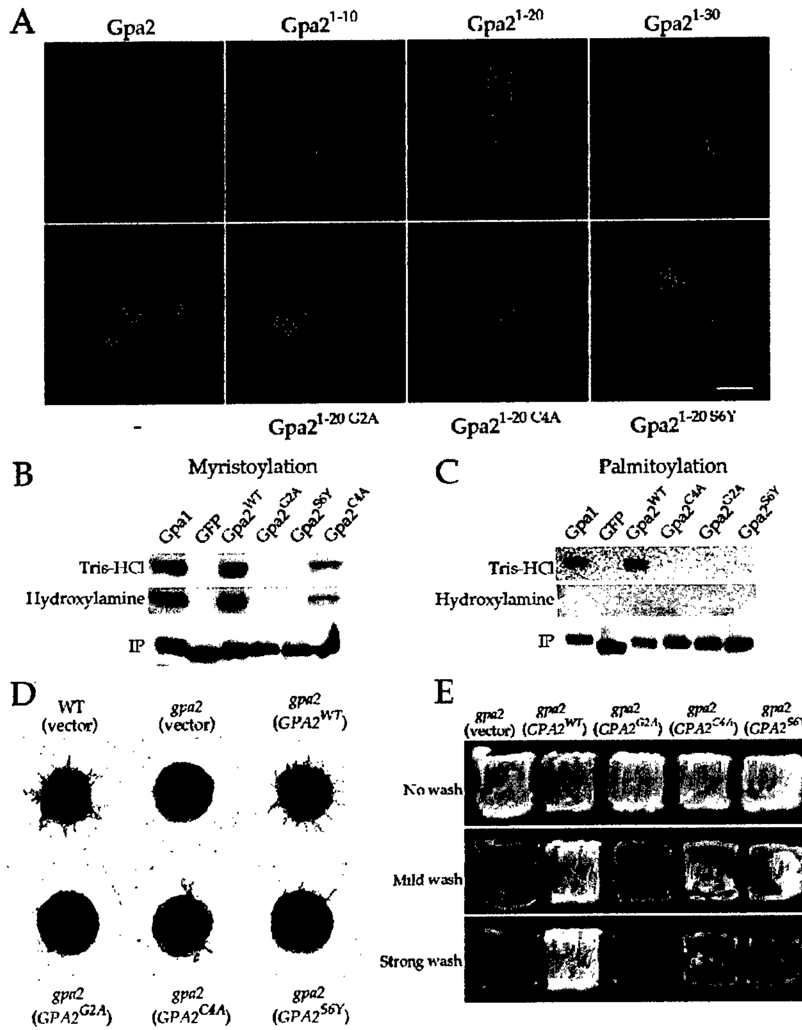


Figure 2. Myristoylation and palmitoylation are required for membrane localization and function of the Gα subunit Gpa2. (A) The first 10 amino acids from Gpa2 are sufficient for membrane localization. A functionally, internally GFP-tagged Gpa2 (Gpa2, pTH80), truncated GFP-tagged Gpa2 proteins, $Gpa2^{1-10}$-GFP ($Gpa2^{1-10}$, pTH71), $Gpa2^{1-20}$-GFP-FLAG ($Gpa2^{1-20}$, pTH81), and $Gpa2^{1-30}$-GFP ($Gpa2^{1-30}$, pTH65), or mutant truncated GFP-tagged Gpa2 proteins, $Gpa2^{1-20\ G2A}$-GFP-FLAG ($Gpa2^{1-20\ G2A}$, pTH91), $Gpa2^{1-20\ C4A}$-GFP-FLAG ($Gpa2^{1-20\ C4A}$, pTH92), and $Gpa2^{1-20\ S6Y}$-GFP-FLAG ($Gpa2^{1-20\ S6Y}$, pTH93), were expressed from a 2μ plasmid in wild-type yeast cells (MLY61a/α) to test for protein localization. The GFP cassette alone (−, pTH73) was also expressed as a control. Scale bar, 5 μm. (B and C) Gpa2 is myristoylated (B) and palmitoylated (C). *gpa2* mutant cells (MLY132a/α) expressing the $Gpa2^{1-20}$-GFP-FLAG ($Gpa2^{WT}$, pTH81), $Gpa2^{1-20\ G2A}$-GFP-FLAG ($Gpa2^{G2A}$, pTH91), $Gpa2^{1-20\ S6Y}$-GFP-FLAG ($Gpa2^{S6Y}$, pTH93), $Gpa2^{1-20\ C4A}$-GFP-FLAG ($Gpa2^{C4A}$, pTH92), GFP-FLAG (GFP, pTH100), or $Gpa1^{1-20}$-GFP-FLAG (Gpa1, pTH103) proteins were metabolically labeled with [^{3}H]myristic acid or [^{3}H]palmitic acid. FLAG-tagged proteins were purified using anti-FLAG affinity gel and subjected to SDS-PAGE. Gels were treated with 1 M Tris-HCl, 1 M hydroxylamine that cleaves the palmitoyl moiety of fatty acids, or subjected to Western blot using an anti-FLAG antibody to verify purified protein levels. Radiolabeled purified proteins were visualized by autoradiography. (D and E) Myristoylation and palmitoylation are required for Gpa2 function. Full-length wild-type ($Gpa2^{WT}$, pTH47) or mutant Gpa2 proteins ($Gpa2^{G2A}$ (pTH62), $Gpa2^{C4A}$ (pTH68), and $Gpa2^{S6Y}$ (pTH69)) were expressed in *gpa2* mutant cells (MLY132a/α or MLY132α) to test for diploid filamentous growth (D) and haploid invasive growth (E). *gpa2* mutant cells containing an empty plasmid (pTH19) served as control.

Gpa2 Binding Partners Are Not Required for Gpa2 Membrane Localization

In heterotrimeric G proteins, Gβγ subunits can promote membrane localization of their associated Gα subunits. Therefore, the localization of Gpa2 was examined in the absence of Gpb1/2 or when Gpb1/2 were overexpressed. As shown in Figure 3, A and B, Gpa2 membrane localization was unchanged under both conditions. Furthermore, deletion of other known Gpa2 associated proteins, namely the GPCR Gpr1 or the Gγ subunit mimic Gpg1, or even the elimination of multiple binding partners (Gpb1/2 and Gpr1 or Gpb1/2 and Gpg1), did not perturb Gpa2 plasma membrane localization, suggesting these binding partners are not required for membrane targeting (Figure 3A).

F3

Because Gpa2 is a component of the glucose sensing cAMP signaling pathway and the agonist induced redistribution of Gαs has been reported in mammalian cells (Wedegaertner *et al.*, 1996; Thiyagarajan *et al.*, 2002), we examined if carbon source affects Gpa2 protein localization (Figure 3C). Glucose serves as a ligand for Gpr1 (Yun *et al.*, 1998; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Rolland *et al.*, 2000; Lemaire *et al.*, 2004). Glucose, fructose, and galactose are structurally related hexoses, yet galactose is not a ligand for Gpr1 (Lorenz *et al.*, 2000; Lemaire *et al.*, 2004). Fructose is controversial, although fructose can induce cAMP production when added to glucose-starved cells (Yun *et al.*, 1998; Lemaire *et al.*, 2004). Maltose and galactose induce filamentous growth in a Gpr1-Gpa2-independent manner (Lorenz *et al.*, 2000). Ethanol and glycerol are structurally unrelated nonfermentable carbon sources. As shown in Figure 3C, Gpa2 was localized to the plasma membrane to the same extent under all conditions tested. Therefore, the carbon sources examined do not influence Gpa2 protein localization and Gpa2 is localized to the cell membrane irrespective of activity of the Gpr1-Gpa2 signaling pathway.

Kelch Gβ Mimic Gpb2 Is Recruited to the Plasma Membrane by Gpa2

If the kelch proteins Gpb1/2 function as Gβ mimics, we hypothesized that Gpb1/2 should also be membrane localized. To examine protein localization, a functional GFP-Gpb2 protein was expressed in *gpa2*Δ cells (Figure 4). When GFP-Gpb2 was expressed alone, Gpb2 was found to be cytoplasmic. However, when GFP-Gpb2 was coexpressed with either wild-type Gpa2 or a dominant negative Gpa2 ($Gpa2^{G299A}$), GFP-Gpb2 was directed to the plasma mem-

F4

balt2/zmk-mbc/zmk-mbc/zmk01005/zmk7325-05a | eldeg | S=9 | 9/2/05 | 9:29 | Art | Input-clp

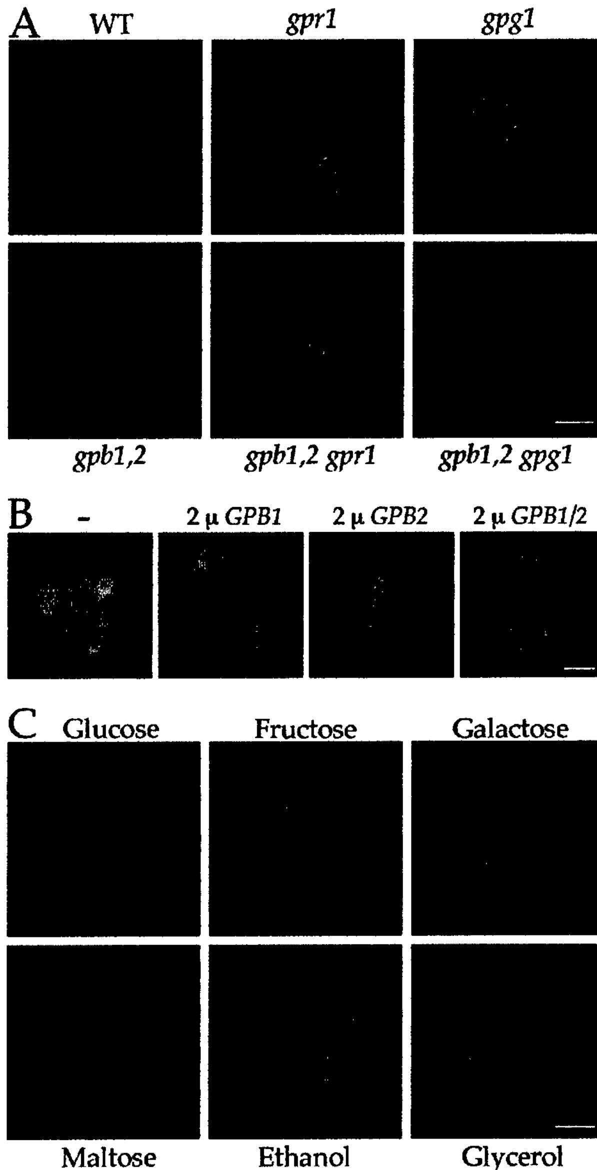


Figure 3. The Gα subunit Gpa2 is localized to the plasma membrane independent of its known binding partners. (A) Gpa2-GFP protein (pTH80) was expressed in *gpr1* (MLY232a/α), *gpg1* (THY224a/α), *gpb1,2* (THY212a/α), *gpb1,2 gpr1* (THY243a/α), and *gpb1,2 gpg1* (THY246a/α) mutant cells and protein localization was analyzed. (B) Overexpression of the kelch Gβ mimic proteins Gpb1/2 has no effect on Gpa2 membrane localization. The Gpa2-GFP protein was coexpressed with Gpb1 (pTH26), Gpb2 (pTH27), or both (pTH26 and pTH114) in wild-type cells (MLY97a/α). (C) Membrane localization of Gpa2 was not altered by carbon sources. *gpa2* mutant cells (MLY132a/α) expressing the Gpa2-GFP protein were grown in synthetic media containing different carbon sources and Gpa2 protein localization was assessed. Scale bars, 5 μm.

brane (Figure 4). Confocal microscopic analysis revealed that Gpb2 was localized to the plasma membrane more extensively when coexpressed with the Gpa2^{G299A} mutant protein that is unable to undergo the GTP-induced conformational change when compared with wild-type Gpa2 (Fig-

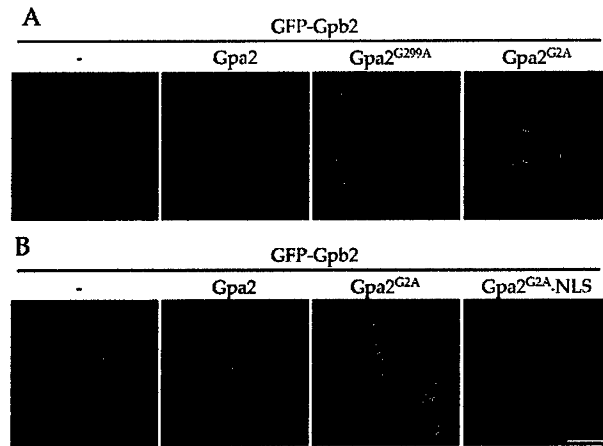


Figure 4. Gα subunit Gpa2 recruits the kelch Gβ subunit mimic Gpb2 to the plasma membrane. (A) A functional GFP-Gpb2 protein (pTH84) was coexpressed with Gpa2 (pTH47), Gpa2^{G299A} (pTH49), Gpa2^{G2A} (pTH62), or Gpa2^{G2A}-NLS (pTH149) proteins in *gpa2Δ* mutant cells (MLY212a/α), and protein localization was investigated by confocal (A) or direct fluorescence microscopy (B). The empty vector pTH19 (−) served as control. Nuclear localization was confirmed by DAPI staining (unpublished data). Scale bar, 5 μm.

ure 4A). This finding is in accord with previous data showing that Gpb2 binds to Gpa2 in vivo and preferentially associates with Gpa2-GDP (Harashima and Heitman, 2002).

When GFP-Gpb2 was coexpressed with the nonfunctional Gpa2^{G2A} mutant that is no longer directed to the plasma membrane, GFP-Gpb2 was no longer localized to the plasma membrane (Figure 4). To exclude the possibility that the observed Gpb2 membrane localization is an indirect secondary consequence due to overexpression of the functional wild-type Gpa2 protein, GFP-Gpb2 was coexpressed with a nuclear localization signal (NLS) containing Gpa2^{G2A} mutant protein (Gpa2^{G2A}-NLS). Strikingly, Gpa2^{G2A}-NLS now misdirected Gpb2 to the nucleus (Figure 4B). Therefore, the Gα protein Gpa2 forms a stable complex with the kelch Gβ mimic protein Gpb2 and serves to recruit Gpb2 to the plasma membrane. That Gpa2^{G2A}-NLS directs Gpb2 to the nucleus also demonstrates that lipid modifications are not required for the Gpa2-Gpb2 interaction. This is consistent with findings regarding interaction of the yeast Gα subunit Gpa1 and the mammalian Gα subunit Gαi with their respective Gβ subunits (Jones *et al.*, 1990; Song *et al.*, 1996).

Kelch Gβ Mimic Gpb2 and the C-terminal Tail of the Gpr1 Receptor Bind to the N-terminal Region of Gpa2

In canonical Gα subunits, an N-terminal alpha helix called the αN domain provides a binding surface for the Gβ subunit and the coupled receptor (Lambright *et al.*, 1996; Wall *et al.*, 1998). Because the αN domain is less conserved among Gα subunits, we searched for any related alpha helical domain in the extended N-terminus of Gpa2 using the PHD secondary structure prediction method (Rost and Sander, 1993). A sequence spanning amino acid residues 49–57 was identified that is predicted to form an alpha helix, although this region does not share any significant identity with known αN domains (Figure 1).

To examine if this candidate alpha helical domain of Gpa2 is involved in the interaction with Gpb2, the domain was deleted in the dominant negative Gpa2^{G299A} mutant

balt2/zmk-mbc/zmk-mbc/zmk01005/zmk7325-05a | eldeg | S=9 | 9/2/05 | 9:29 | Art | input-clp

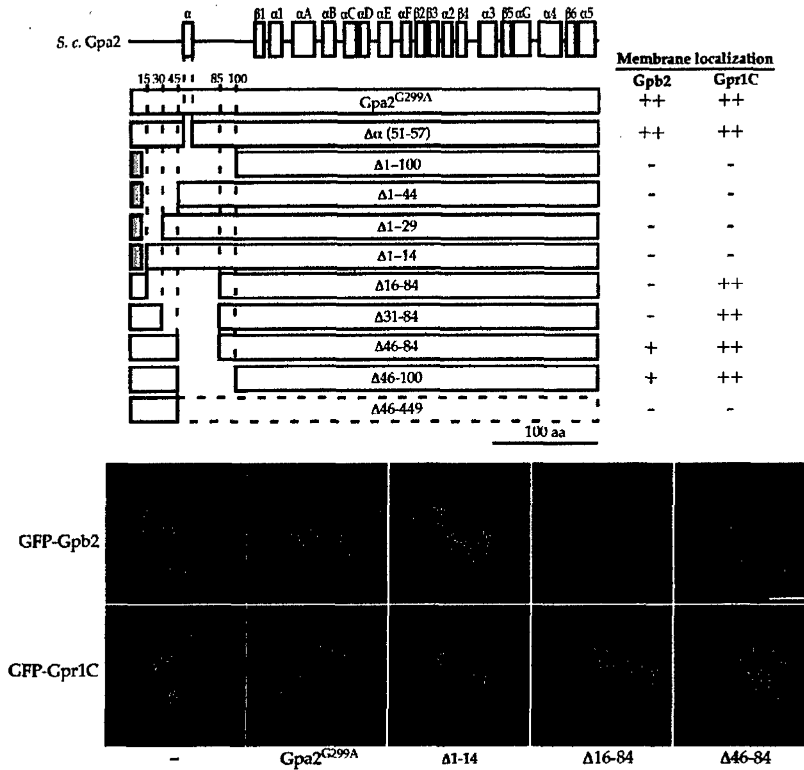


Figure 5. N-terminus of Gα Gpa2 is required for binding to the kelch protein Gpb2 and the GPCR Gpr1. A series of deletions was created in the N-terminal region of Gpa2^{G299A}, and these deletion constructs were coexpressed with the GFP-Gpb2 protein (pTH84) in *gpa2*Δ cells (MLY212a/α) or with GFP-Gpr1C (pTH170) in wild-type cells (S1338) to determine roles of the N-terminal region of Gpa2 on interaction with Gpb2 and the C-terminal tail of Gpr1. Deletion mutant Gpa2^{G299A} proteins constructed and results are shown schematically. Δ1–14, Δ1–29, Δ1–44, and Δ1–100 mutant proteins were fused to the first 10 amino acids from the yeast Gα subunit Gpa1 to restore targeting to the plasma membrane and Gpa1 residues are depicted as a gray box. Scale bar, 5 μm.

(Gpa2$^{\Delta\alpha\ (51-57)}$) and the resulting mutant derivative was coexpressed with the GFP-Gpb2 protein to test for protein localization. As noted above, Gpa2^{G299A} recruits GFP-Gpb2 to the plasma membrane (Figure 5). Similarly, Gpa2$^{\Delta\alpha\ (51-57)}$ also brought GFP-Gpb2 to the plasma membrane (Figure 5). Therefore, the sequence spanning amino acids 51–57, which is predicted to be an N-terminal alpha helical region, is not required for Gpa2-Gpb2 binding.

We next addressed whether other sequences in the Gpa2 N-terminal extension are required for Gpb2 interaction. For this purpose, deletions were introduced into the N-terminal region of the *GPA2*^{G299A} allele to create Δ1–14, Δ1–29, Δ1–44, and Δ1–100 derivatives of Gpa2^{G299A}, which were also then fused to the first 10 amino acids from the *S. cerevisiae* Gα subunit Gpa1 that are sufficient for membrane localization (unpublished data; Gillen *et al.*, 1998). Internal deletions were also created (Δ16–84, Δ31–84, Δ46–84, and Δ46–100, Figure 5). This deletion mutant series was coexpressed with GFP-Gpb2 to examine which Gpa2 mutants are capable of recruiting GFP-Gpb2 to the plasma membrane (Figure 5). All deletions generated for this study (except for the Δ46–449 Gpa2 mutant) are predicted to have no significant impact on the secondary structure of Gpa2, based on PHD analysis, and the function and expression of these alleles of *GPA2*^{G299A} were confirmed by introducing these alleles into wild-type diploid cells and examining pseudohyphal growth (unpublished data). All deletion constructs and representative results are shown in Figure 5.

GFP-Gpb2 did not associate with the plasma membrane when coexpressed with the Δ1–14, Δ1–29, Δ1–44, or Δ1–100 Gpa2 derivatives, indicating that the N-terminus of Gpa2 plays an important role in Gpb2 binding (Figure 5). However, the first 15 or 30 amino acids were not sufficient for Gpb2 binding because neither the Gpa2 Δ16–84 nor the Δ31–84 mutant was able to recruit Gpb2 to the plasma membrane. On the other hand, membrane localization of GFP-Gpb2 was observed when it was coexpressed with the Gpa2 Δ46–84 and Δ46–100 mutants. Taken together, these findings indicate that the first 45 amino acids are necessary for Gpb2 interaction. This N-terminal region alone (1–45 aa) was not sufficient because GFP-Gpb2 was cytoplasmic with the Gpa2$^{\Delta 46-449}$ variant. Structural analyses have revealed that Gβ binding interfaces are present not only in the N-terminus (the αN domain) but also in the central region (β2 to α2 domain) of conventional Gα molecules (Figure 1 and Lambright *et al.*, 1996; Wall *et al.*, 1998). Therefore, by analogy Gpa2 may also require the corresponding internal conserved region in conjunction with the N-terminal 1–45 aa to bind Gpb2, although we cannot exclude a possibility that the Gpa2$^{\Delta 46-449}$ variant failed to recruit Gpb2 to the plasma membrane because of instability. Note that the deletions examined were also introduced into a wild-type Gpa2 construct and tested for GFP-Gpb2 interaction as above, and results were essentially equivalent to the ones with the Gpa2^{G299A} deletion variants with the minor difference that plasma membrane localization of GFP-Gpb2 was weaker when the wild-type Gpa2 deletion variant were coexpressed. This is consistent with the fact that Gpa2^{G299A} binds to Gpb2 more strongly than does wild-type Gpa2 (Figure 4, Harashima and Heitman, 2002, 2004).

We next addressed regions of the Gpa2 molecule involved in association with the Gpr1 receptor. Previously, the Gpr1 C-terminal tail composed of 99 amino acids was isolated in a yeast two-hybrid screen that identified Gpa2 interacting proteins (Xue *et al.*, 1998). Because Gpr1 that is C-terminally tagged with GFP is nonfunctional (unpublished data), likely because of interference with Gpr1-Gpa2 coupling, we fused GFP to the N-terminus of the 99 amino acid soluble C-

terminal tail of Gpr1. The resulting GFP fusion protein (GFP-Gpr1C) was coexpressed with the Gpa2^{G299A} variants to examine roles of the N-terminal extension on interactions with the coupled receptor Gpr1, as above (Figure 5, also see Figure 8).

As shown in Figure 5, any variant of Gpa2 lacking the first 15 amino acids failed to recruit GFP-Gpr1C to the plasma membrane (Gpa2$^{\Delta 1-14}$, Gpa2$^{\Delta 1-29}$, Gpa2$^{\Delta 1-44}$, and Gpa2$^{\Delta 1-100}$), whereas all of the variants containing amino acids 1–15 (Gpa2$^{\Delta 16-84}$, Gpa2$^{\Delta 31-84}$, Gpa2$^{\Delta 46-84}$, and Gpa2$^{\Delta 46-100}$) recruited GFP-Gpr1C, similar to full length Gpa2^{G299A}. The only exception was Gpa2$^{\Delta 46-449}$, which failed to recruit the GFP-Gpr1C to the plasma membrane. These observations indicate that the N-terminal region of Gpa2 participates in associating with the receptor C-terminal tail, but that C-terminal regions of Gpa2 likely also participate. Importantly, the C-terminal tail of other Gα subunits is known to be involved in receptor coupling (Slessareva *et al.*, 2003; Herrmann *et al.*, 2004). Consistent with this model, Gpa2$^{\Delta 1-100}$ still interacted with the C-terminal tail of Gpr1 in the yeast two-hybrid assay and Gpa2 function was perturbed by a C-terminal GFP tag (unpublished data). In summary, these data indicate that both the N-terminal and more C-terminal regions of the Gα protein Gpa2 are required for interactions with both Gpb2 and Gpr1.

Functional Roles of the Gpa2 N-terminus

To address roles of the Gpa2 amino terminus, N-terminal deletions were introduced into wild-type Gpa2. The resulting deletion alleles were expressed in diploid or haploid *gpa2* mutant cells to examine whether these mutants complement *gpa2* defects in pseudohyphal growth, invasive growth, and glucose-induced cAMP production (Figure 6). These mutant alleles were also introduced into diploid *gpr1 gpa2* mutant cells to examine whether they require Gpr1 for function or act as dominant alleles that bypass the receptor. Cells expressing Gpa2$^{\Delta 1-100}$ exhibited reduced pseudohyphal and invasive growth and reduced levels of basal and glucose-induced cAMP, indicating that the N-terminal region plays an important functional role or that deletion of the 1–100 amino acids might result in misfolding of Gpa2 (Figures 6). Gpa2$^{\Delta 46-84}$, Gpa2$^{\Delta 46-100}$, and Gpa2$^{\Delta \alpha\ (51-57)}$ all functioned as wild-type Gpa2, likely because Gpb2 and the C-terminal tail of Gpr1 still bind to these deletion proteins (Figure 6 and unpublished data). The Δ1–14, Δ1–29, Δ1–44, Δ16–84, or Δ31–84 *GPA2* mutant genes were largely able to complement *gpa2* mutant phenotypes. One interpretation of these results is that these deletion proteins still functionally interact with Gpr1 and Gpb2 via other Gpa2 domains and are capable of functioning, similar to wild-type Gpa2. Or expression of the deletion Gpa2 proteins from a multicopy plasmid might mask their reduced activity so that expression from a low copy plasmid could elicit altered mutant phenotypes. Alternatively, these results could be due to counterbalancing defects in Gpa2 interaction with Gpr1 and Gpb2 because Gpr1/Gpa2 and Gpb2 control the cAMP signaling pathway positively and negatively, respectively (see *Discussion*).

Kelch Gβ Mimic Proteins Gpb1/2 Function on the Plasma Membrane

Gpb2 is directed to the plasma membrane in a Gpa2 dependent manner, indicating that the kelch Gβ mimic proteins Gpb1/2 may function on the plasma membrane. To examine this hypothesis, the first 10 amino acids of Gpa2 (hereafter, the membrane localization sequence [MLS]) that suffice for membrane localization were fused to the N-terminus of the GFP-Gpb1 or GFP-Gpb2 protein. The resulting fusion proteins were tested for protein localization and complementation of the elevated filamentous phenotype of *gpb1,2* mutant cells (Figure 7). We also tested the effects of fusing a nuclear localization signal (NLS) from the SV40 T antigen to the N-terminus of the GFP-Gpb1 or GFP-Gpb2 protein (Figure 7).

The MLS- and NLS-fused GFP-Gpb1/2 proteins were predominantly localized to the plasma membrane and the nucleus, respectively (Figure 7A). Furthermore, the MLS-GFP-Gpb1/2 fusion proteins complemented the *gpb1,2* double mutant phenotype and restored wild-type pseudohyphal growth (Figure 7B). In contrast, the nuclear localized Gpb1/2 proteins (NLS-GFP-Gpb1/2) were nonfunctional (Figure 7B). These findings provide evidence that Gpb1/2 can function when heterologously targeted to the plasma membrane. These results also indicate that the as yet unidentified second target of Gpb1/2 might be membrane associated.

Kelch Gβ Mimic Proteins Gpb1/2 Inhibit Gpr1-Gpa2 Coupling

Gpa2 interacts with the C-terminal tail of the Gpr1 receptor and recruits the GFP-Gpr1 C-tail fusion protein to the plasma membrane. Here we used this assay to analyze Gpr1-Gpa2 coupling in further detail. GFP-Gpr1C is localized to the plasma membrane when coexpressed with the dominant negative Gpa2^{G299A} allele. Additionally, membrane localization of GFP-Gpr1C was less pronounced when coexpressed with wild-type Gpa2, suggesting that the C-terminal tail of Gpr1 binds more strongly to Gpa2^{G299A} compared to wild-type Gpa2 (Figure 8). On the other hand, interaction of Gpa2 with the C-terminal tail of Gpr1 was reduced even further with the dominant Gpa2^{Q300L} allele (Figure 8). This is consistent with the widely accepted model in which the Gα-GDP complex binds to the cognate GPCR, whereas the Gα-GTP complex dissociates from the GPCR. To confirm the interaction between GFP-Gpr1C and Gpa2, the nonfunctional nuclear localized Gpa2^{G2A}-NLS was coexpressed with GFP-Gpr1C. In this case, GFP-Gpr1C was now misdirected to the nucleus (Figure 8).

Because Gpb2 is directed to the plasma membrane in a Gpa2-dependent manner and binds to the N-terminus of Gpa2 where the C-terminal tail of Gpr1 also binds, we hypothesized that Gpb1/2 could negatively regulate Gpa2 function by inhibiting the Gpr1-Gpa2 interaction. To address this hypothesis, the wild-type Gpb1/2 proteins were simultaneously coexpressed with the GFP-Gpr1C and Gpa2^{G299A} proteins. As shown in Figure 8, the membrane localization of GFP-Gpr1 was significantly reduced by coexpression of Gpb1/2, indicating that Gpb1/2 compete with the C-terminal tail of Gpr1 for binding to the N-terminus of Gpa2. Gpb1/2 may thereby control Gpa2 function by impairing receptor coupling. This is in contrast to canonical Gβ subunits, which function to promote interactions of the Gα subunit with the associated GPCR.

DISCUSSION

The Roles of the N-terminal Region of Gpa2

The MG^{2}XXXS^{6} sequence in open reading frames and the glycine residue of the consensus sequence are well defined as a myristoylation consensus sequence and the myristoylation site. On the other hand, no obvious consensus sequence is established for palmitoylation, yet palmitoylation mostly occurs in a cysteine residue(s) near the N-terminus.

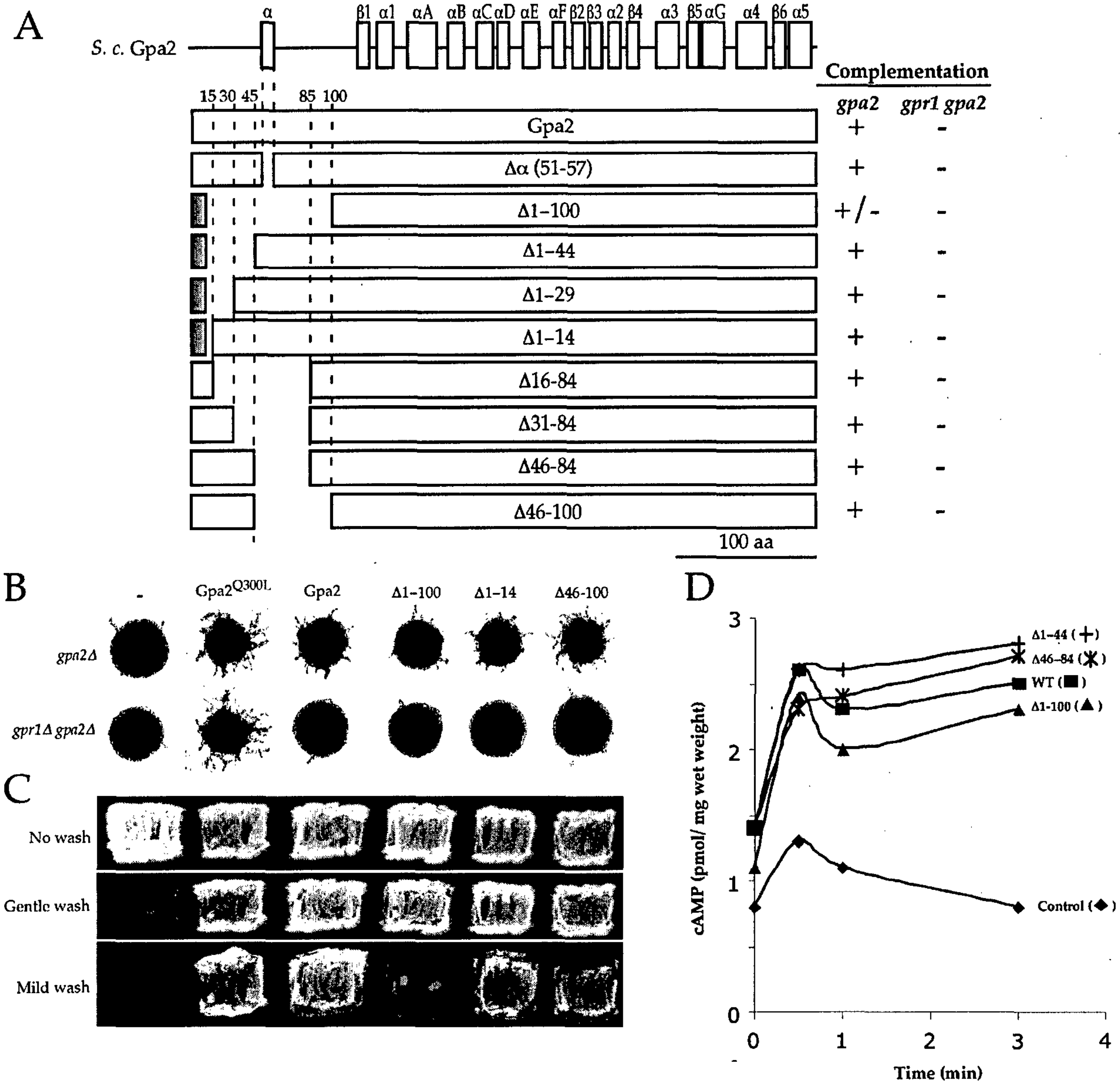


Figure 6. Function of the N-terminal deletion Gpa2 proteins in vivo. (A) Schematic of N-terminal deletion Gpa2 variants and complementation results in *gpa2* or *gpr1 gpa2* mutant cells. N-terminal deletions were created in the wild-type *GPA2* gene and introduced into *gpa2* (MLY132α for invasive growth assay and MLY132a/α for pseudohyphal growth assay) or *gpr1 gpa2* (MLY277a/α) mutant cells and ability to complement pseudohyphal and invasive growth defects was examined. Representative data are shown in B for pseudohyphal growth and in C for invasive growth. (D) Glucose-induced cAMP production in *gpa2* (MLY132α) mutant cells expressing the N-terminal deletion Gpa2 derivatives. The values shown are the mean of two independent experiments, except the control, which is representative of cells carrying the empty vector (pTH19).

The Gα subunit Gpa2 contains the MG[2]XXXS[6] myristoylation consensus sequence and a cysteine at the fourth position of its N-terminus. A cysteine after the N-terminal cysteine appears at the 189th position of the Gpa2 protein. Our biochemical studies revealed that Gpa2 is myristoylated and palmitoylated. Furthermore, the labeling and site-directed mutagenesis studies shown in Figure 2 provide evidence that Gpa2 is myristoylated at Gly[2] and, most likely, also palmitoylated at Cys[4].

Introduction of site-specific mutations (G2A, C4A, and S6Y) into the *GPA2* and *GPA2-GFP* fusion genes demonstrates that myristoylation and palmitoylation are critical for plasma membrane targeting and function of Gpa2. Although it still remains to be established why myristoylation is essential for Gα function, recent studies demonstrate that GPCR-Gα fusion proteins, in which Gα is localized to the plasma membrane yet no longer lipid modified, are functional in vivo (for review, see Seifert *et al.*, 1999). Furthermore, a nonmyristoylated Gαi2^{Q205L} protein is unable to signal and fails to transform rat fibroblasts (Gallego *et al.*, 1992). Consistently, we also found that a nonmyristoylated dominant Gpa2^{Q300L} mutant (equivalent to Gαi2 Q205L) is incapable of enhancing filamentous growth in wild-type cells. These findings support a model in which lipid modi-

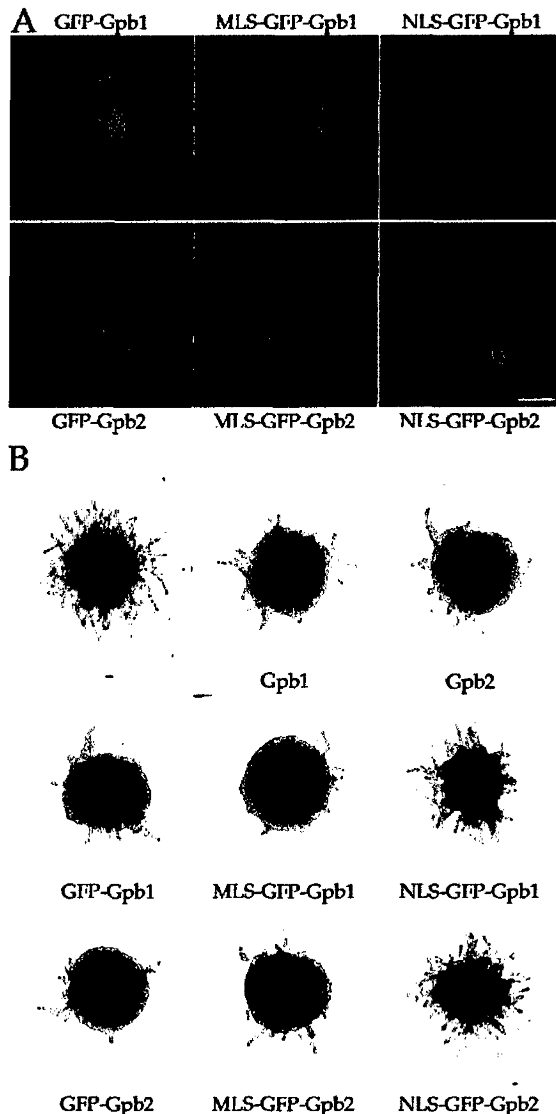


Figure 7. Kelch Gβ mimic proteins Gpb1/2 function on the plasma membrane. A membrane localization sequence (MLS) or nuclear localization signal (NLS) was fused to the N-terminus of the functional GFP-Gpb1/2 proteins (pTH106/pTH75) and the resulting fusion proteins (pTH163, pTH164, pTH166, or pTH167) were expressed in diploid *gpb1,2* double mutant cells (THY212a/α) to test for protein localization (A) and function (B). The MLS-GFP-Gpb1/2 fusion proteins were recruited to the plasma membrane and were as functional as the wild-type Gpb1/2 proteins, whereas the NLS-GFP-Gpb1/2 fusion proteins were directed to the nucleus and nonfunctional. Cells bearing the empty vector (pTH19) or the *GPB1* (pTH26) or *GPB2* (pTH27) plasmid served as controls. Scale bar, 5 μm.

fications are necessary for plasma membrane targeting that is a prerequisite for Gα function. Alternatively, myristoylation may play an important role in Gα structure that is required for receptor coupling (Preininger *et al.*, 2003).

In heterotrimeric G proteins, the N-terminus is also involved in interactions with Gβγ dimer, receptors, and effectors. Structural and biochemical studies implicate the N-terminal alpha helix (αN domain) in Gβγ dimer and receptor coupling (Lambright *et al.*, 1996; Wall *et al.*, 1998). Gpa2 contains an alpha helix in the extended N-terminus, yet the position of this helix is not conserved (Figure 1). More strikingly, the alpha helix is not involved in coupling to the kelch subunit Gpb2 or to the Gpr1 C-terminal tail. Studies using Gpa2 variants that carry a series of deletions in the Gpa2 N-terminus identified binding domains for the Gpr1 C-terminal tail and Gpb2 that map to amino acids 1–15 and 1–45 and are not predicted to form an alpha helix.

Lipid modifications alone are not sufficient to restore these interactions as the Gpa2 Δ1–14 mutant that is lipid modified on an appended $Gpa1^{1-10}$ peptide did not direct the binding partners to the plasma membrane. Rather, amino acid sequences that lie between residues 1–45 are important for the interactions. Interestingly, the non-alpha helical N-terminus (spanning amino acids 1–6) of Gαq is known to be involved in receptor selectivity (Kostenis *et al.*, 1997). Therefore, the N-terminus may play a direct role in receptor coupling by providing a binding interface or an indirect role by influencing overall structure. Either possibility is novel and further studies, especially structural studies, should address the role of the N-terminus of Gpa2.

The Role of the Gpr1 C-terminal Tail

Previous studies suggest the presence of preactivation complexes in which an unoccupied, inactive GPCR is coupled to the Gα subunit (Samama *et al.*, 1993; Stefan *et al.*, 1998; Dosil *et al.*, 2000). Such preactivation complexes are not necessarily required for formation of the activated ternary complex in which a ligand bound, activated receptor forms a complex with a G protein to stimulate GDP-GTP exchange on Gα, yet the preactivation complexes are involved in regulation of specificity and intensity of G-protein mediated signaling (Neubig, 1994; Shea and Linderman, 1997). In *S. cerevisiae*, the C-terminal tail of the α-factor receptor Ste2 is implicated in the formation of the preactivation complex with its associated Gα Gpa1 (Dosil *et al.*, 2000). Although no direct evidence has been reported for a preactivation complex between the Gpr1 receptor and Gpa2, our data support the existence of one. First, the cytoplasmic C-terminal tail of Gpr1 binds to wild-type Gpa2 and a nuclear localized $Gpa2^{G2A}$-NLS. Second, Gpr1 and Gpa2 are still functional in the absence of the Gβ mimic subunits Gpb1/2, suggesting a promiscuous coupling between Gpr1 and Gpa2.

These observations may be relevant to our finding that N-terminal deletion variants of Gpa2 (Δ1–14, Δ1–29, Δ1–44, and Δ1–100) that are unable to bind to the Gpr1 C-terminal tail are still functional and can respond to glucose to stimulate cAMP production. This interpretation may also explain why cells expressing these Gpa2 variants exhibited near wild-type phenotypes. It is conceivable that a reduced affinity of the Gpa2 variants with the Gpr1 receptor could result in a decrease in signaling leading to a low-PKA phenotype. However, these Gpa2 variants also show decreased binding to the kelch subunits Gpb1/2 that negatively control cAMP signaling, affecting Gpb1/2 function to activate the as yet unidentified second target that inhibits cAMP signaling.

Kelch Subunits Gpb1/2 Inhibit Gpr1-Gpa2 Coupling

G-protein activity is controlled at multiple steps including expression, protein localization, GDP-GTP exchange, and GTPase activity. GPCRs activate G proteins by stimulating GDP dissociation from Gα and acting as guanine nucleotide exchange factors, thereby leading to Gα in the active Gα-GTP form. On the other hand, the GoLoco family protein AGS3 functions as a guanine nucleotide dissociation inhibitor (GDI) by inhibiting GDP-GTP exchange (De Vries *et al.*, 2000). Although GoLoco homologues are conserved in mul-

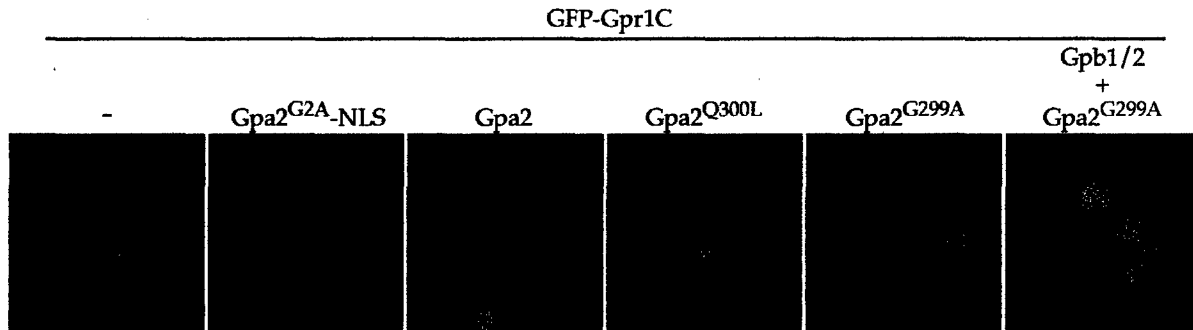


Figure 8. Kelch Gβ mimic proteins Gpb1/2 interfere with the interaction between Gpa2 and the C-terminal tail of Gpr1. (A) The GFP-Gpr1C fusion protein (pTH170) was expressed alone or coexpressed with Gpa2 variants, wild-type Gpa2 (pTH47), Gpa2^{Q300L} (pTH48), Gpa2^{G299A} (pTH49), or NLS-Gpa2^{G2A} (pTH149) with or without Gpb1/2 (pTH174/pTH114) in wild-type cells (THY452). Empty vectors (pTH171 and pTH173) were used as controls for the Gpb1/2 plasmids, pTH174 and pTH114. The location of nuclei were confirmed by DAPI staining.

ticellular eukaryotes, no such homolog is apparent in the yeast genome.

Our previous studies revealed that the kelch subunits Gpb1 and Gpb2 negatively control Gpa2 and preferentially associate with Gpa2-GDP (Harashima and Heitman, 2002). However, neither loss nor overexpression of Gpb1/2 perturbed Gpa2 membrane localization or expression. In addition, Gpb1/2 did not exhibit GDI activity under standard in vitro conditions (unpublished data). Here we show that Gpb1/2 inhibit Gpa2-Gpr1 coupling. A model governing how the kelch Gpb1/2 subunits control Gpa2 is that Gpb1/2 bind to the Gpa2 N-terminal region spanning amino acids 1–45 and occlude binding of the Gpr1 C-terminal tail to the first fifteen amino acids of Gpa2 (Figure 9).

In canonical heterotrimeric G proteins, G$\beta\gamma$ subunits are required for receptor-Gα coupling. In *S. cerevisiae*, the G$\beta\gamma$ dimer plays an essential role in pheromone receptor-Gα Gpa1 coupling (Blumer and Thorner, 1990). In mammalian systems, a role for the G$\beta\gamma$ subunits in coupling of β_2-adrenergic receptor-Gαs, M_2-muscarinic receptor-Gαo, A_1-adenosine and 5-HT_{1A} receptors-Gαi, and β_2-adrenergic receptor-Gαi has been established (Richardson and Robishaw, 1999; Hou *et al.*, 2001; Lim *et al.*, 2001; Kühn *et al.*, 2002). This function is opposite to the role of the kelch subunits, yet

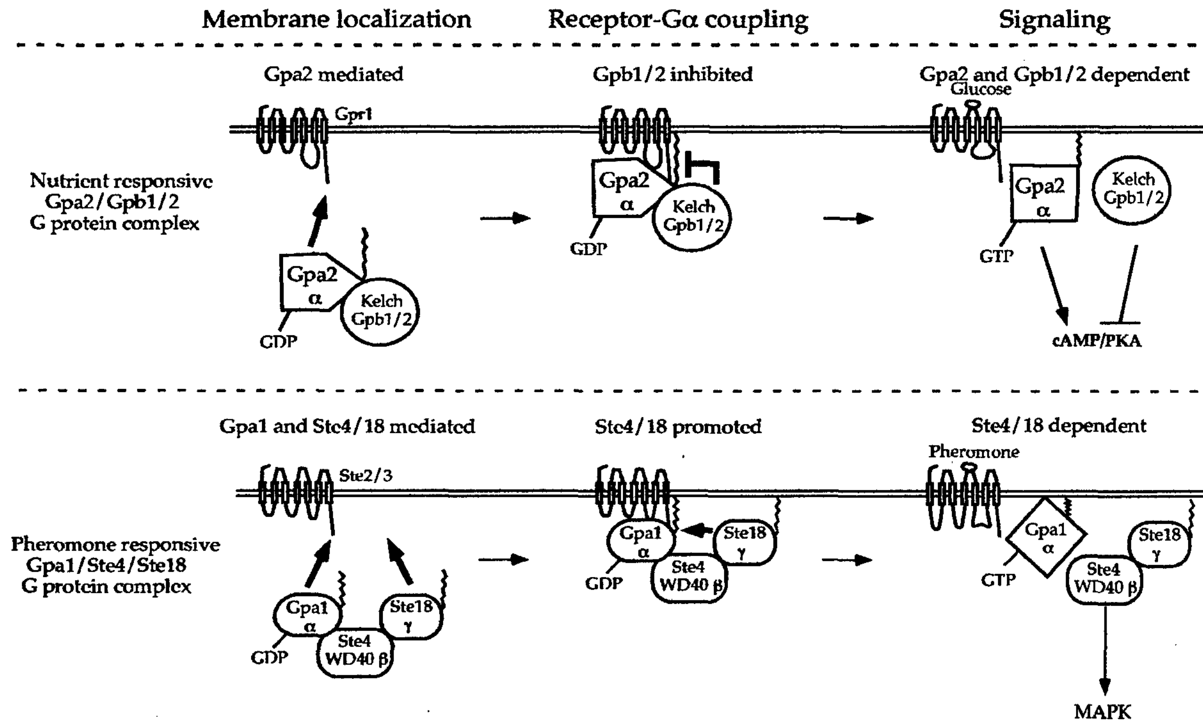


Figure 9. Model of canonical heterotrimeric and atypical G protein signaling in budding yeast. The canonical heterotrimeric G protein composed of the Gpa1/Ste4/Ste18 subunits regulates the pheromone responsive MAPK cascade, whereas the atypical heteromeric G protein consisting of the Gpa2/Gpb1/2 subunits controls the nutrient sensing cAMP-PKA signaling pathway. For details, see *Discussion*.

importantly, yeast and mammalian WD40 repeat $G\beta\gamma$ subunits and the kelch subunits all converge to modulate receptor-$G\alpha$ coupling. That receptor-$G\alpha$ coupling is oppositely regulated may depend on how tightly and specifically a given $G\alpha$ binds to its associated receptor. In yeast, the pheromone receptor Ste2 is functionally coupled to the $G\alpha$ protein Gpa1 and not to the Gpa2 $G\alpha$ subunit (Blumer and Thorner, 1990). During diploid filamentation, the glucose receptor Gpr1 is associated with Gpa2 and not with the haploid specific $G\alpha$ Gpa1. Importantly, the Gpa2 $G\alpha$ subunit is still partially functional and able to signal in response to the agonist glucose via Gpr1 in the absence of Gpb1/2, suggesting that Gpa2 can functionally couple to its receptor in the absence of Gpb1/2 (Harashima and Heitman, 2002). Therefore, Gpa2 may normally be tightly associated with the Gpr1 receptor, and Gpb1/2 function to compete with this association to reduce signaling in the absence of glucose.

Generally, the intracellular third loop of GPCRs plays a crucial role in interactions with the $G\alpha$ subunit. Although *S. cerevisiae* Gpa2 has been reported to interact with the intracellular third loop of Gpr1 in the yeast two-hybrid assay (Yun *et al.*, 1997), we were unable to recapitulate this result (unpublished data). This could be attributable to a weak interaction between Gpa2 and the third loop of Gpr1. In contrast, the Gpr1 C-terminal tail avidly binds to Gpa2 in two-hybrid assays (Yun *et al.*, 1997; Xue *et al.*, 1998; Kraakman *et al.*, 1999; Harashima and Heitman, 2002). We also showed that the Gpa2-Gpr1 C-terminal tail interaction can be detected using the GFP tagged C-terminal tail of Gpr1 in vivo (Figures 5 and 8). These data indicate that the Gpr1 C-terminus plays an important role in Gpa2 binding. This atypical feature of the Gpr1 receptor-Gpa2 $G\alpha$ complex may mirror the unusual aspects by which the kelch subunits Gpb1/2 inhibit the signaling complex.

Is Gpa2 an Unusual $G\alpha$ or an Ancestral $G\alpha$ Subunit?

Our studies provide evidence that lipid modifications (myristoylation and palmitoylation) of $G\alpha$ Gpa2 are necessary and sufficient for Gpa2 plasma membrane targeting but are not required for interaction with the kelch $G\beta$ mimic subunit Gpb2. Instead, Gpa2 directs Gpb2 to the plasma membrane. Mammalian $G\alpha$ subunits as well as the yeast canonical $G\alpha$ subunit Gpa1 share similar features. Like Gpa2, lipid modifications but not the $G\beta\gamma$ dimer are required for plasma membrane localization of yeast Gpa1 and mammalian $G\alpha$ (Song *et al.*, 1996; Gillen *et al.*, 1998; Galbiati *et al.*, 1999). It has also been reported that a nonlipidated $G\alpha$ still binds to $G\beta\gamma$ subunits in yeast and mammals (Jones *et al.*, 1990; Degtyarev *et al.*, 1994; Song *et al.*, 1996). Studies also provide evidence that $G\alpha$, at least in part, directs $G\beta\gamma$ subunits to the plasma membrane in vivo (Song *et al.*, 1996; Takida and Wedegaertner, 2003). Although Gpa2 shares similar features with canonical $G\alpha$ subunits, a striking contrast is the inability of Gpa2 to form a heterotrimeric G protein. The $G\alpha$ subunit Gpa1 in the fission yeast *Schizosaccharomyces pombe*, which functions in pheromone-mediated signaling, also fails to form a heterotrimeric G protein with the known $G\beta\gamma$ subunits Git5/11. The kelch protein Ral2 has been proposed as a possible Gpa1-associated subunit based on genetic studies (Fukui *et al.*, 1989; Harashima and Heitman, 2002; Hoffman, 2005).

Another contrast between canonical $G\alpha$ subunits and Gpa2 is that $G\beta\gamma$ subunits typically promote receptor-$G\alpha$ coupling, whereas Gpb1/2 inhibit receptor-Gpa2 coupling (Figure 9). The receptor Gpr1 and $G\alpha$ Gpa2 can still in part function and signal in response to glucose without the $G\beta$ mimic subunits Gpb1/2, indicating a promiscuous and specific coupling between Gpr1 and Gpa2 even in the absence of Gpb1/2 (Harashima and Heitman, 2002). In *S. cerevisiae*, the cAMP-PKA signaling pathway is essential for cell growth and determines cell fates in response to extracellular nutrients (Harashima and Heitman, 2004). Therefore the cAMP-PKA signaling pathway should be strictly controlled, and for this reason, Gpb1/2 may interfere with promiscuous Gpr1-Gpa2 coupling to facilitate responses to extracellular nutrients. On the other hand, in canonical G proteins, the $G\beta\gamma$ dimer may control $G\alpha$ function by increasing the specificity of receptor coupling (Richardson and Robishaw, 1999; Hou *et al.*, 2001; Lim *et al.*, 2001; Kühn *et al.*, 2002). Importantly, the kelch $G\beta$ mimic subunits Gpb1/2 and canonical $G\beta\gamma$ dimer both regulate receptor-$G\alpha$ coupling. Thus, the Gpa2/Gpb1/2 protein complex shares features with canonical heterotrimeric G proteins, and we propose Gpa2 is an ancestral subunit rather than an unusual $G\alpha$ subunit. In this model, eukaryotic cells first acquired a GPCR and associated $G\alpha$ subunit to sense and signal extracellular cues. Later, seven-bladed β-propeller-type subunits (kelch or WD40 based) were recruited to the GPCR-$G\alpha$ signaling complex. Finally, farnesylated $G\gamma$ subunits were recruited to promote membrane localization. In this model, the atypical features of the nutrient and pheromone GPCR-$G\alpha$ signaling modules in budding and fission yeasts might mirror features of their ancestral signaling modules from which they derive.

Alternatively, yeasts might uniquely have evolved an "alternative" $G\alpha$ subunit and established a novel G protein signaling system to sense extracellular stimuli, in which an atypical $G\alpha$ subunit forms a complex and functions with an unusual binding-partner kelch $G\beta$ mimic protein. Further studies in both unicellular and multicellular organisms would distinguish these possibilities.

ACKNOWLEDGMENTS

We thank Sayoko Ito-Harashima for providing a yeast strain and Cristl Arndt and Emily Wenink for assistance. We also thank Yong-Sun Bahn, Alex Idnurm, Julian Rutherford, Chaoyang Xue, Andy Alspaugh, Pat Casey, Henrik Dohlman, and Bob Lefkowitz for critical reading. This study was supported by the Department of Defense Neurofibromatosis program (W81xwh-04-01-0208). T.H. was supported by a fellowship from the Children's Tumor Foundation and J.H. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

Adams, J., Kelso, R., and Cooley, L. (2000). The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol. *10*, 17–24.

Arévalo-Rodríguez, M., and Heitman, J. (2005). Cyclophilin A is localized to the nucleus and controls meiosis in *Saccharomyces cerevisiae*. Eukaryot. Cell *4*, 17–29.

Ashrafi, K., Farazi, T. A., and Gordon, J. I. (1998). A role for *Saccharomyces cerevisiae* fatty acid activation protein 4 in regulating protein N-myristoylation during entry into stationary phase. J. Biol. Chem. *273*, 25864–25874.

Batlle, M., Lu, A. L., Green, D. A., Xue, Y., and Hirsch, J. P. (2003). Krh1p and Krh2p act downstream of the Gpa2p $G\alpha$ subunit to negatively regulate haploid invasive growth. J. Cell Sci. *116*, 701–710.

Blumer, K. J., and Thorner, J. (1990). β and γ subunits of a yeast guanine nucleotide-binding protein are not essential for membrane association of the α subunit but are required for receptor coupling. Proc. Natl. Acad. Sci. USA *87*, 4363–4367.

Bourne, H. R. (1997). How receptors talk to trimeric G proteins. Curr. Opin. Cell Biol. *9*, 134–142.

Cabrera-Vera, T. M., Vanhauwe, J., Thomas, T. O., Medkova, M., Preininger, A., Mazzoni, M. R., and Hamm, H. E. (2003). Insights into G protein structure, function, and regulation. Endocr. Rev. *24*, 765–781.

Chen, C. A., and Manning, D. R. (2001). Regulation of G proteins by covalent modification. Oncogene *20*, 1643–1652.

Colombo, S. *et al.* (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae.* EMBO J. *17*, 3326–3341.

De Vries, L., Fischer, T., Tronchère, H., Brothers, G. M., Strockbine, B., Siderovski, D. P., and Farquhar, M. G. (2000). Activator of G protein signaling 3 is a guanine dissociation inhibitor for $G\alpha_i$ subunits. Proc. Natl. Acad. Sci. USA *97*, 14364–14369.

De Vries, L., and Gist Farquhar, M. (1999). RGS proteins: more than just GAPs for heterotrimeric G proteins. Trends Cell Biol. *9*, 138–144.

Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. (1994). Palmitoylation of a G protein α_i subunit requires membrane localization not myristoylation. J. Biol. Chem. *269*, 30898–30903.

Dohlman, H. G. (2002). G proteins and pheromone signaling. Annu. Rev. Physiol. *64*, 129–152.

Dohlman, H. G., and Thorner, J. W. (2001). Regulation of G protein-initiated signal transduction in yeast: paradigms and principles. Annu. Rev. Biochem. *70*, 703–754.

Dosil, M., Schandel, K. A., Gupta, E., Jenness, D. D., and Konopka, J. B. (2000). The C terminus of the *Saccharomyces cerevisiae* α-factor receptor contributes to the formation of preactivation complexes with its cognate G protein. Mol. Cell. Biol. *20*, 5321–5329.

Evanko, D. S., Thiyagarajan, M. M., and Wedegaertner, P. B. (2000). Interaction with $G\beta\gamma$ is required for membrane targeting and palmitoylation of $G\alpha_s$ and $G\alpha_q$. J. Biol. Chem. *275*, 1327–1336.

Farazi, T. A., Waksman, G., and Gordon, J. I. (2001). The biology and enzymology of protein *N*-myristoylation. J. Biol. Chem. *276*, 39501–39504.

Fishburn, C. S., Pollitt, S. K., and Bourne, H. R. (2000). Localization of a peripheral membrane protein: $G\beta\gamma$ targets $G\alpha_Z$. Proc. Natl. Acad. Sci. USA *97*, 1085–1090.

Fukui, Y., Miyake, S., Satoh, M., and Yamamoto, M. (1989). Characterization of the *Schizosaccharomyces pombe ral2* gene implicated in activation of the *ras1* gene product. Mol. Cell. Biol. *9*, 5617–5622.

Galbiati, F., Volonté, D., Meani, D., Milligan, G., Lublin, D. M., Lisanti, M. P., and Parenti, M. (1999). The dually acylated NH_2-terminal domain of $G_{i1\alpha}$ is sufficient to target a green fluorescent protein reporter to caveolin-enriched plasma membrane domains. Palmitoylation of caveolin-1 is required for the recognition of dually acylated G-protein α subunits *in vivo.* J. Biol. Chem. *274*, 5843–5850.

Gallego, C., Gupta, S. K., Winitz, S., Eisfelder, B. J., and Johnson, G. L. (1992). Myristoylation of the $G\alpha_{i2}$ polypeptide, a G protein α subunit, is required for its signaling and transformation functions. Proc. Natl. Acad. Sci. USA *89*, 9695–9699.

Gancedo, J. M. (2001). Control of pseudohyphae formation in *Saccharomyces cerevisiae.* Fems Microbiol. Rev. *25*, 107–123.

Gautam, N., Downes, G. B., Yan, K., and Kisselev, O. (1998). The G-protein $\beta\gamma$ complex. Cell Signal *10*, 447–455.

Gillen, K. M., Pausch, M., and Dohlman, H. G. (1998). N-terminal domain of Gpa1 (G protein α subunit) is sufficient for plasma membrane targeting in yeast *Saccharomyces cerevisiae.* J. Cell Sci. *111*, 3235–3244.

Gilman, A. G. (1987). G-Proteins: transducers of receptor-generated signals. Annu. Rev. Biochem. *56*, 615–649.

Guan, K. L., and Han, M. (1999). A G-protein signaling network mediated by an RGS protein. Genes Dev. *13*, 1763–1767.

Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988). Site of G protein binding to rhodopsin mapped with synthetic peptides from the α subunit. Science *241*, 832–835.

Harashima, T., and Heitman, J. (2002). The $G\alpha$ protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic $G\beta$ subunits. Mol. Cell *10*, 163–173.

Harashima, T., and Heitman, J. (2004). Nutrient control of dimorphic growth in *Saccharomyces cerevisiae.* In: Topics in Current Genetics, Vol. 7, ed. J Winderickx and P. M. Taylor, Heidelberg: Springer-Verlag, 131–169.

Herrmann, R., Heck, M., Henklein, P., Henklein, P., Kleuss, C., Hofmann, K. P., and Ernst, O. P. (2004). Sequence of interactions in receptor-G protein coupling. J. Biol. Chem. *279*, 24283–24290.

Hoffman, C. S. (2005). Except in every detail: comparing and contrasting G-protein signaling in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* Eukaryot. Cell *4*, 495–503.

Hou, Y., Chang, V., Capper, A. B., Taussig, R., and Gautam, N. (2001). G Protein β subunit types differentially interact with a muscarinic receptor but not adenylyl cyclase type II or phospholipase C-β2/3. J. Biol. Chem. *276*, 19982–19988.

Ito, N., Phillips, S. E., Stevens, C., Ogel, Z. B., McPherson, M. J., Keen, J. N., Yadav, K. D., and Knowles, P. F. (1991). Novel thioether bond revealed by a 1.7 Å crystal structure of galactose oxidase. Nature *350*, 87–90.

Ito, N., Phillips, S.E.V., Yadav, K.D.S., and Knowles, P. F. (1994). Crystal structure of a free radical enzyme, galactose oxidase. J. Mol. Biol. *238*, 794–814.

Jeansonne, N. E. (1994). Yeast as a model system for mammalian seven-transmembrane segment receptors. Proc. Soc. Exp. Biol. Med. *206*, 35–44.

Johnson, D. R., Bhatnagar, R. S., Knoll, L. J., and Gordon, J. I. (1994). Genetic and biochemical studies of protein N-myristoylation. Annu. Rev. Biochem. *63*, 869–914.

Jones, T. L., Simonds, W. F., Merendino, J. J., Jr., Brann, M. R., and Spiegel, A. M. (1990). Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. Proc. Natl. Acad. Sci. USA *87*, 568–572.

Journot, L., Pantaloni, C., Bockaert, J., and Audigier, Y. (1991). Deletion within the amino-terminal region of $G_s\alpha$ impairs its ability to interact with $\beta\gamma$ subunits and to activate adenylate cyclase. J. Biol. Chem. *266*, 9009–9015.

Kostenis, E., Degtyarev, M. Y., Conklin, B. R., and Wess, J. (1997). The N-terminal extension of $G\alpha_q$ is critical for constraining the selectivity of receptor coupling. J. Biol. Chem. *272*, 19107–19110.

Kraakman, L., Lemaire, K., Ma, P. S., Teunissen, A.W.R.H., Donaton, M.C.V., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999). A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol. Microbiol. *32*, 1002–1012.

Kübler, E., Mösch, H. U., Rupp, S., and Lisanti, M. P. (1997). Gpa2p, a G-protein α-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J. Biol. Chem. *272*, 20321–20323.

Kühn, B., Christel, C., Wieland, T., Schultz, G., and Gudermann, T. (2002). G-protein $\beta\gamma$-subunits contribute to the coupling specificity of the β_2-adrenergic receptor to G_s. Naunyn Schmiedebergs Arch. Pharmacol. *365*, 231–241.

Lambright, D. G., Sondek, J., Bohm, A., Skiba, N. P., Hamm, H. E., and Sigler, P. B. (1996). The 2.0 Å crystal structure of a heterotrimeric G protein. Nature *379*, 311–319.

Lefkowitz, R. J. (2000). The superfamily of heptahelical receptors. Nat. Cell Biol. *2*, E133–E136.

Lemaire, K., Van de Velde, S., Van Dijck, P., and Thevelein, J. M. (2004). Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae.* Mol. Cell *16*, 293–299.

Lengeler, K. B., Davidson, R. C., D'Souza, C., Harashima, T., Shen, W. C., Wang, P., Pan, X. W., Waugh, M., and Heitman, J. (2000). Signal transduction cascades regulating fungal development and virulence. Microbiol. Mol. Biol. Rev. *64*, 746–785.

Lim, W. K., Myung, C. S., Garrison, J. C., and Neubig, R. R. (2001). Receptor-G protein γ specificity: γ11 shows unique potency for A_1 adenosine and $5\text{-}HT_{1A}$ receptors. Biochemistry *40*, 10532–10541.

Linder, M. E., Pang, I. H., Duronio, R. J., Gordon, J. I., Sternweis, P. C., and Gilman, A. G. (1991). Lipid modifications of G protein subunits. Myristoylation of $G_{o\alpha}$ increases its affinity for $\beta\gamma$. J. Biol. Chem. *266*, 4654–4659.

Longtine, M. S., McKenzie III, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae.* Yeast *14*, 953–961.

Lorenz, M. C., and Heitman, J. (1997). Yeast pseudohyphal growth is regulated by GPA2, a G protein α homolog. EMBO J. *16*, 7008–7018.

Lorenz, M. C., Pan, X. W., Harashima, T., Cardenas, M. E., Xue, Y., Hirsch, J. P., and Heitman, J. (2000). The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae.* Genetics *154*, 609–622.

Mombaerts, P. (2004). Genes and ligands for odorant, vomeronasal and taste receptors. Nat. Rev. Neurosci. *5*, 263–278.

Morales, J., Fishburn, C. S., Wilson, P. T., and Bourne, H. R. (1998). Plasma membrane localization of $G\alpha_z$ requires two signals. Mol. Biol. Cell *9*, 1–14.

Navon, S. E., and Fung, B. K. (1987). Characterization of transducin from bovine retinal rod outer segments. Participation of the amino-terminal region of T_α in subunit interaction. J. Biol. Chem. *262*, 15746–15751.

Neubig, R. R. (1994). Membrane organization in G-protein mechanisms. FASEB J. *8*, 939–946.

Pan, X., Harashima, T., and Heitman, J. (2000). Signal transduction cascades regulating pseudohyphal differentiation of *Saccharomyces cerevisiae.* Curr. Opin. Microbiol. *3,* 567–572.

Preininger, A. M., Van Eps, N., Yu, N. J., Medkova, M., Hubbell, W. L., and Hamm, H. E. (2003). The myristoylated amino terminus of $G\alpha_{i1}$ plays a critical role in the structure and function of $G\alpha_{i1}$ subunits in solution. Biochemistry *42,* 7931–7941.

Richardson, M., and Robishaw, J. D. (1999). The α_{2A}-adrenergic receptor discriminates between G_i heterotrimers of different $\beta\gamma$ subunit composition in Sf9 insect cell membranes. J. Biol. Chem. *274,* 13525–13533.

Rolland, F., de Winde, J. H., Lemaire, K., Boles, E., Thevelein, J. M., and Winderickx, J. (2000). Glucose-induced cAMP signalling in yeast requires both a G-protein coupled receptor system for extracellular glucose detection and a separable hexose kinase-dependent sensing process. Mol. Microbiol. *38,* 348–358.

Ross, E. M., and Wilkie, T. M. (2000). GTPase-activating proteins for heterotrimeric G proteins: Regulators of G protein signaling (RGS) and RGS-like proteins. Annu. Rev. Biochem. *69,* 795–827.

Rost, B., and Sander, C. (1993). Prediction of protein secondary structure at better than 70% accuracy. J. Mol. Biol. *232,* 584–599.

Samama, P., Cotecchia, S., Costa, T., and Lefkowitz, R. J. (1993). A mutation-induced activated state of the β_2-adrenergic receptor. Extending the ternary complex model. J. Biol. Chem. *268,* 4625–4636.

Schwartz, M. A., and Madhani, H. D. (2004). Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae.* Annu. Rev. Genet. *38,* 725–748.

Schwindinger, W. F., and Robishaw, J. D. (2001). Heterotrimeric G-protein $\beta\gamma$-dimers in growth and differentiation. Oncogene *20,* 1653–1660.

Seifert, R., Wenzel-Seifert, K., and Kobilka, B. K. (1999). GPCR-Gα fusion proteins: molecular analysis of receptor-G-protein coupling. Trends Pharmacol. Sci. *20,* 383–389.

Shea, L., and Linderman, J. J. (1997). Mechanistic model of G-protein signal transduction. Determinants of efficacy and effect of precoupled receptors. Biochem. Pharmacol. *53,* 519–530.

Sherman, F. (1991). Getting started with yeast. Methods Enzymol. *194,* 3–21.

Slessareva, J. E., Ma, H., Depree, K. M., Flood, L. A., Bae, H., Cabrera-Vera, T. M., Hamm, H. E., and Graber, S. G. (2003). Closely related G-protein-coupled receptors use multiple and distinct domains on G-protein α-subunits for selective coupling. J. Biol. Chem. *278,* 50530–50536.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E., and Sigler, P. B. (1996). Crystal structure of a G_A protein $\beta\gamma$ dimer at 2.1 Å resolution. Nature *379,* 369–374.

Song, J., and Dohlman, H. G. (1996). Partial constitutive activation of pheromone responses by a palmitoylation-site mutant of a G protein α subunit in yeast. Biochemistry *35,* 14806–14817.

Song, J., Hirschman, J., Gunn, K., and Dohlman, H. G. (1996). Regulation of membrane and subunit interactions by N-myristoylation of a G protein α subunit in yeast. J. Biol. Chem. *271,* 20273–20283.

Sprang, S. R. (1997). G protein mechanisms: Insights from structural analysis. Annu. Rev. Biochem. *66,* 639–678.

Stefan, C. J., Overton, M. C., and Blumer, K. J. (1998). Mechanisms governing the activation and trafficking of yeast G protein-coupled receptors. Mol. Biol. Cell *9,* 885–899.

Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994). Structure and function of G protein-coupled receptors. Annu. Rev. Biochem. *63,* 101–132.

Takida, S., and Wedegaertner, P. B. (2003). Heterotrimer formation, together with isoprenylation, is required for plasma membrane targeting of $G\beta\gamma$. J. Biol. Chem. *278,* 17284–17290.

Tamaki, H., Miwa, T., Shinozaki, M., Saito, M., Yun, C. W., Yamamoto, K., and Kumagai, H. (2000). GPR1 regulates filamentous growth through *FLO11* in yeast *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. *267,* 164–168.

Thiyagarajan, M. M., Bigras, E., Van Tol, H. H., Hébert, T. E., Evanko, D. S., and Wedegaertner, P. B. (2002). Activation-induced subcellular redistribution of $G\alpha_s$ is dependent upon its unique N-terminus. Biochemistry *41,* 9470–9484.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22,* 4673–4680.

Wall, M. A., Coleman, D. E., Lee, E., Iñiguez-Lluhi, J. A., Posner, B. A., Gilman, A. G., and Sprang, S. R. (1995). The structure of the G protein heterotrimer $G_{i\alpha1\beta1\gamma2}$. Cell *83,* 1047–1058.

Wall, M. A., Posner, B. A., and Sprang, S. R. (1998). Structural basis of activity and subunit recognition in G protein heterotrimers. Structure *6,* 1169–1183.

Wedegaertner, P. B., Bourne, H. R., and von Zastrow, M. (1996). Activation-induced subcellular redistribution of $G_{s\alpha}$. Mol. Biol. Cell *7,* 1225–1233.

Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., and Bourne, H. R. (1993). Palmitoylation is required for signaling functions and membrane attachment of $G_q\alpha$ and $G_s\alpha$. J. Biol. Chem. *268,* 25001–25008.

Wilson, P. T., and Bourne, H. R. (1995). Fatty acylation of a_z. Effects of palmitoylation and myristoylation on α_z signaling. J. Biol. Chem. *270,* 9667–9675.

Wise, A., Grassie, M. A., Parenti, M., Lee, M., Rees, S., and Milligan, G. (1997). A cysteine-3 to serine mutation of the G-protein $G_{i1}\alpha$ abrogates functional activation by the α_{2A}-adrenoceptor but not interactions with the $\beta\gamma$ complex. Biochemistry *36,* 10620–10629.

Xue, Y., Batlle, M., and Hirsch, J. P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p G_α subunit and functions in a Ras-independent pathway. EMBO J. *17,* 1996–2007.

Yamaguchi, Y., Katoh, H., and Negishi, M. (2003). N-terminal short sequences of α subunits of the G_{12} family determine selective coupling to receptors. J. Biol. Chem. *278,* 14936–14939.

Yun, C. W., Tamaki, H., Nakayama, R., Yamamoto, K., and Kumagai, H. (1997). G-protein coupled receptor from yeast *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. *240,* 287–292.

Yun, C. W., Tamaki, H., Nakayama, R., Yamamoto, K., and Kumagai, H. (1998). Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. *252,* 29–33.