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Direct Activation of Fission Yeast Adenylyl Cyclase by Heterotrimeric G Protein gpa2

HIDEKI OGIHARA[1], FUMI SHIMA[1], KANA NAITO[1], TSUYOSHI ASATO[2], KEN-ICHI KARIYA[2], and TOHRU KATAOKA[1]

[1]*Division of Molecular Biology, Department of Molecular and Cellular Biology, Kobe University Graduate School of Medicine*

[2]*Division of Cell Biology, Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan*

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Genetic studies on *Schizosaccharomyces pombe* adenylyl cyclase (cyr1) have shown that its activity is positively regulated by a heterotrimetric G protein α subunit gpa2 and that the resulting increase in intracellular cAMP concentration causes inhibition of sexual development including mating and meiosis. However, molecular mechanism underlying this gpa2-dependent regulation of cyr1 remains to be clarified. Here, we show that gpa2 exhibits a direct and GTP-dependent binding to the Ras-associating domain (RAD) of cyr1, which is identified by a computer algorithm-based search of the cyr1 amino acid sequence. Overexpression of this RAD results in acceleration of the sexual development of fission yeast cells presumably by competitive sequestration of gpa2. Furthermore, cyr1 is activated *in vitro* by the addition of purified gpa2, which is converted to the active state by treatment with AlF_4^-. These results indicate a crucial role of the RAD as a direct binding site of gpa2 in activation of cyr1. Thus, RADs, which have been defined as a conserved motif shared among the Ras-family small G protein-associating domains, are for the first time shown to exhibit a functional association with a member of the heterotrimeric G proteins.

Heterotrimetric G proteins consist of α, β and γ subunits, and regulate numerous signal transduction pathways in eukaryotic cells (26, 34). In the GDP-bound inactive state, Gα subunit is associated with Gβγ dimer to form a heterotrimeric complex. G protein-coupled receptors, after ligand binding, stimulate GDP-GTP exchange on Gα and formation of its GTP-bound active state, which also results in its dissociation from Gβγ (7, 20, 34). In the active state, Gα-GTP and Gβγ are free to interact with and regulate downstream effectors, including adenylyl cyclase, phospholipase C, mitogen-activated protein kinase cascades, and ion channels (8, 26).

The fission yeast *Schizosaccharomyses (S.) pombe* possesses two heterotrimeric Gα subunits, gpa1 and gpa2 (27). gpa1 couples to the membrane-associated pheromone receptor and regulates the activity of a mitogen-activated protein kinase cascade of the mating pheromone pathway in cooperation with a small G protein ras1, a homologue of mammalian Ras oncoprotein (4, 43). On the other hand, gpa2 is implicated in regulation of adenylyl cyclase (cyr1) in response to nutritional condition (2, 11, 14, 22, 23). In *S. pompe*, nutritional conditions are known to affect the sexual development. The intracellular cAMP level is lowered when cells are subjected to nitrogen starvation (4, 22), while increase in the cAMP

Phone: 81-78-382-5380 Fax: 81-78-382-5399 E-mail: kataoka@kobe-u.ac.jp

level inhibits mating and meiosis, primarily by repressing expression of the genes required for the sexual development (1, 40). Thus, regulation of the cyr1 activity appears to be highly relevant to the initiation of sexual development. cyr1 consists of 1,693-amino acid residues (44, 45) and is composed of at least two domains: the N-terminal regulatory and C-terminal catalytic domains. Previous genetic studies have revealed that several *git* genes, including *git3* (encoding a glucose receptor), *gpa2/git8*, *git5* (Gβ subunit) and *git11* (Gγ subunit) are required for proper regulation of the cyr1 activity (18, 19, 23, 41). Disruption of *gpa2* causes constitutive mating and sporulation in homothallic strains, reduction in the basal cAMP levels, and elimination of the glucose-induced cAMP response. git5 and git11, which form a complex *in vivo* (18), were also shown to be required for the glucose-induced cAMP response. However, because the defect in glucose response associated with the disruption of *git5* or *git11* was suppressed by gpa2 overexpression, it is likely that git5 and git11 are involved in positive regulation of cyr1 through the gpa2 activation (18, 19). Although the formation of a trimeric complex among gpa2, git5, and git11 has not been proved yet, the git5/git11 dimer may be required for translocation of the gpa2/git5/git11 complex to the plasma membrane, where activated receptors stimulate transition of gpa2 to the GTP-bound active state (19). These results support a crucial role of gpa2 in the cyr1 regulation. However, little is known about its underlying molecular mechanism.

In the budding yeast *Saccharomyces* (*S.*) *cerevisiae*, adenylyl cyclase is directly regulated by small G proteins RAS1 and RAS2 (38). Recently, we demonstrated that GTP-bound Ras directly associate with the Ras-associating domain (RAD) of adenylyl cyclase to achieve its activation (16). RAD is a motif of about 100-amino acid residues which is conserved among the known Ras/Rap1/M-Ras-binding sites of various effector proteins (5, 21, 30, 35). Recent X-ray crystallographic studies revealed that overall tertiary structure of Ral-GDS-RAD is quite similar to those of the Ras-binding domains (RBDs) of Raf-1 and phosphoinositide 3-kinaseγ even though no extensive sequence similarity is found between RADs and RBDs (6, 12, 13, 24, 25, 29, 39).

In this paper, we report identification of an RAD in *S. pombe* cyr1. Further, we show that this RAD makes a direct association with GTP-bound gpa2 and mediates gpa2-dependent cyr1 activation.

MATERIALS AND METHODS

Yeast Strains and Growth Media

S. pombe strains used in this study are listed in Table I. JZ393 strain (14) was obtained

Table I. Yeast strains used in this study

Strain	Genotype	Extrachromosomal plasmid(s)
JZ393	*h⁻ ade6-M216 leu1 ura4-D18 gpa2 Δ::ura4⁺*	
FS100	*h⁻ ade6-M216 leu1 ura4-D18 gpa2 Δ::ura4⁺*	pREP1-FLAG-cyr1
KN1	*h⁻ ade6-M216 leu1 ura4-D18 gpa2 Δ::ura4⁺*	pREP1-FLAG-gpa2
HO1	*h⁺ ade6-M210 leu1-32*	pREP1-FLAG
HO3	*h⁺ ade6-M210 leu1-32*	pREP1-FLAG-cyr1(242-390)
HO2	*h⁻ leu1-32 ura4-D6*	pREP1-FLAG
HO4	*h⁻ leu1-32 ura4-D6*	pREP1-FLAG-cyr1(242-390)
SHO1	*h⁹⁰ leu1*	pREP1-FLAG
SHO2	*h⁹⁰ leu1*	pREP1-FLAG-cyr1(242-390)

from Dr. Masayuki Yamamoto (University of Tokyo, Tokyo, Japan). pREP1 was obtained from Dr. Takayoshi Kuno (Kobe University Graduate School of Medicine, Kobe, Japan).

Media for routine cultures were prepared as described (9, 28). EMM medium (28) supplemented with auxotrophic nutrients was used for the growth of pREP1-carrying strains, and expression from the *nmt1* promoter was induced by deprivation of thiamine from the medium. Sporulation efficiency was examined in SSA sporulation medium on agar plates (3). Transformation of *S. pombe* cells was performed as described (28).

Construction of Expression Plasmids

pREP1-FLAG vector was constructed by inserting an annealed pair of oligonucleotides, encoding a FLAG epitope (DYKDDDDK), into pREP1. A DNA fragment carrying the full-length cyr1-coding region was cloned into pREP1-FLAG to produce pREP1-FLAG-cyr1 for expression of the full-length cyr1 with a FLAG tag (FLAG-cyr1) under the control of the *nmt1* promoter in yeast cells (Table I). A DNA fragment corresponding to amino acids 242-390 of cyr1, containing the RAD, was cloned into pGEX-2T (Amersham Bioscience) for expression as a fusion with *Schistosoma japonicum* glutathione *S*-transferase (GST) [GST-cyr1(242-390)] in *Escherichia (E.) coli*, or into pREP1-FLAG for expression with a FLAG tag [FLAG-cyr1(242-390)] in yeast. A DNA fragment encoding the full-length gpa2 was cloned into pREP1-FLAG and pFLAG (Sigma) for expression of gpa2 with a FLAG tag (FLAG-gpa2) in *S. pombe* and *E. coli* cells, respectively.

***In Vitro* Binding Assay**

FLAG-gpa2 was affinity-purified from *E. coli* cells harboring pFLAG-gpa2 with resin conjugated with anti-FLAG monoclonal antibody M2 (Sigma). It was preloaded with guanosine 5'-*O*-(2-thiodiphosphate) (GDPβS) or guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) and examined for *in vitro* association with GST-cyr1(242-390), which had been attached to glutathione-Sepharose 4B beads, as described before (32). Bound gpa2 in the eluates from glutathione-Sepharose 4B beads was detected by immunoblotting with anti-FLAG antibody (Sigma). For quantitative *in vitro* association assay, FLAG-gpa2 was loaded with [γ-^{35}S]GTPγS (600 cpm/pmol) or [^{3}H]GDP (600 cpm/pmol), respectively, and incubated with GST-cyr1(242-390) as described above. Bound gpa2 was quantified by counting the eluates from glutathione-Sepharose 4B beads for ^{35}S or ^{3}H label, respectively.

Adenylyl Cyclase Assay

Yeast strain KN1 was cultured in EMM medium. Collected cell pellets were disrupted by shaking with glass beads in buffer B [50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, I mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 1 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride], and the crude membrane fraction was prepared by centrifugation at 27,000 x *g* for 30 min at 4 °C. The posttranslationally modified form of FLAG-gpa2 was extracted from the membrane fraction with buffer B containing 60 mM 1-*O*-*n*-octyl-β-D-glucopyranoside and affinity-purified with resin conjugated with anti-FLAG monoclonal antibody M2. The purified FLAG-gpa2 was treated with 30 μM $AlCl_3$ and 10 mM NaF as described before (17). Crude membrane fraction was prepared from yeast strain FS100, harboring pREP1-FLAG-cyr1 and lacking gpa2, in a similar way except that buffer B was replaced by buffer E [50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.2), 0.1 mM $MgCl_2$, 0.1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, 1mM β-mercaptoethanol, 60 mM 1-*O*-*n*-octyl-β-D-glucopyranoside, 150 mM NaCl, 20 μg/ml aprotinin, and 1 μM leupeptin]. The membrane fraction suspended in buffer E was used for the measurement of adenylyl cyclase activity with the addition of various concentrations of FLAG-gpa2 as described before (33).

RESULTS

Identification of an RAD in cyr1 and Demonstration of its Direct Association with gpa2

Prior to the analysis of gpa2-cyr1 interaction, we searched the whole cyr1 amino acid sequence for the presence of any motif implicated in protein-protein interaction by using Motif Scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan). This search identified an RAD in amino acid 292 to 354 of cyr1, the location comparable to that of an RAD in *S. cerevisiae* adenylyl cyclase (Fig. 1A). X-ray crystallographic analyses of Ral-GDS-RAD and RBDs of Raf-1 and phosphoinositide 3-kinaseγ had revealed that their N-terminal regions, including β1-strand, β2-strand, α1-helix and the following loop, form a critical interface for association with Ras and Rap1 (Fig. 1B). cyr1-RAD contained only 63-amino acid residues and was considerably smaller than various RADs interacting with small G proteins. However,

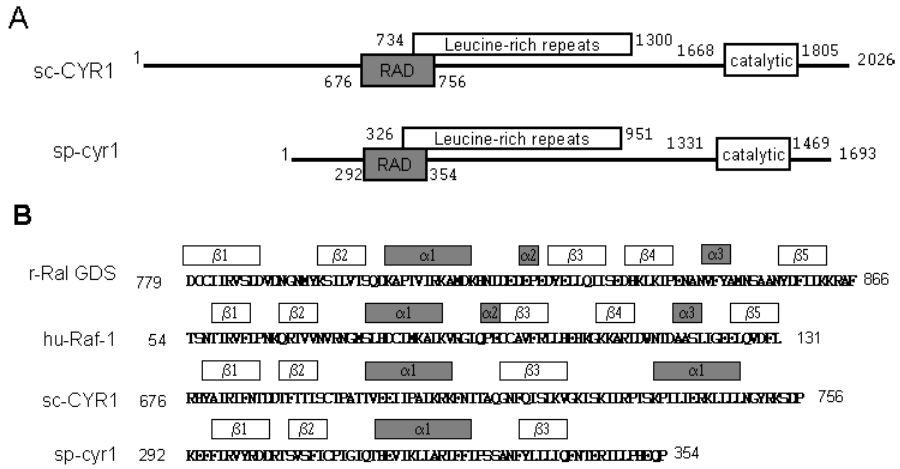

Fig. 1. Comparison of *S. pombe* cyr1-RAD with Ras-binding sites of representative Ras-effector proteins. (A) Domain structures of adenylyl cyclase of *S. cerevisiae* and *S. pombe* adenylyl cyclases are schematically shown. (B) Amino acid sequence alignment of *S. pombe* cyr1-RAD with rat RalGDS-RAD, human Raf-1-RBD, and *S. cerevisiae* adenylyl cyclase (CYR1)-RAD. The secondary structures of sc-CYR1-RAD and sp-cyr1-RAD were predicted by PHD (http://www.embl-heidelberg.de/predictprotein/) derived from the EMBL data base. The secondary structures of RalGDS-RAD and Raf-1-RBD are taken from their tertiary structures (13, 25).

its predicted secondary structure showed a marked resemblance to those of the N-terminal regions of other RADs and RBDs (Fig. 1B).

We examined for direct association of cyr1-RAD with gpa2 *in vitro*. gpa2, which was expressed in *E. coli* and purified, bound to GST-cyr1(242-390) in a GTP-dependent manner (Fig. 2A). This association was further subjected to quantitative measurements using gpa2 loaded with radioisotope-labeled guanine nucleotides (Fig. 2B). gpa2 exhibited a dose-dependent binding to GST-cyr1(242-390) in a GTP-dependent manner. These results indicated that cyr1- RAD constitutes a critical binding site of gpa2.

Overexpression of cyr1-RAD Accelerates the Sexual Development of Fission Yeast Cells

Fission yeast cells expressing a high level of cyr1 grow normally in rich media, but they show different growth properties upon nutritional starvation. After reaching stationary phase, while the wild-type strain undergoes sporulation, they remain relatively sterile and show an elongated cell shape. A similar phenotype is observed in a strain carrying a mutation in *cgs1*,

encoding a regulatory subunit of cAMP-dependent protein kinase (15). Thus, cAMP signaling

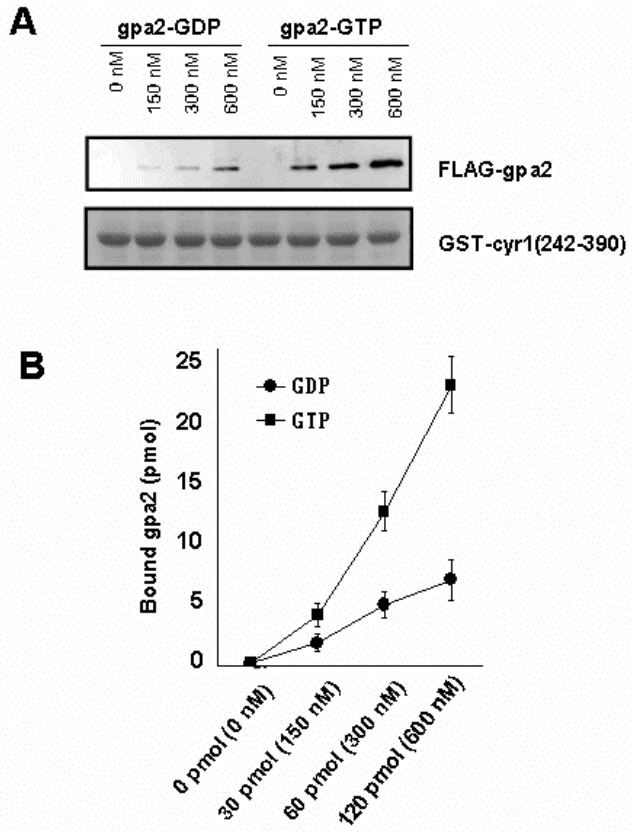

Fig. 2. Measurement of *in vitro* association of gpa2 with cyr1-RAD. (A) 200 pmols of purified GST-cyr1(242-390) were examined for *in vitro* association with various concentrations of purified FLAG-gpa2, which had been preloaded with GDPβS (GDP) or GTPγS (GTP), in 200 μl of the reaction mixtures. GST-cyr1(242-390) and FLAG-gpa2 in the eluate were fractionated by SDS-polyacrylamide gel electrophoresis (10% gel) and detected by staining with Coomassie-Brilliant Blue (lower panel) and by immunoblotting with the anti-FLAG antibody (upper panel), respectively. The experiments were performed three times, yielding equivalent results. (B) Quantitative *in vitro* association assay. Various amounts of FLAG-gpa2, preloaded with [^{35}S]GTPγS or [^{3}H]GDP, were incubated with GST-cyr1(242-390) as described in (A). The amounts of gpa2 bound to cyr1(242-390) were quantitated by counting the eluate for ^{35}S or ^{3}H label. The values are expressed as the means ± S.D. (n = 4).

pathway is implicated in suppression of the sexual differentiation pathway.

In *S. cerevisiae*, overexpression of adenylyl cyclase RAD had been shown to suppress phenotypes associated with an elevated cAMP level because of competitive sequestration of endogenous Ras. By analogy we examined the effect of the cyr1-RAD overexpression in the sexual differentiation of *S. pombe* cells. When cyr1(242-390) was overexpressed in a homothallic strain SHO2, a great increase in sporulation rate was observed upon nutritional starvation (Fig. 3A). An essentially similar result was obtained using heterothallic strains HO3/HO4 (Fig. 3B). These results suggested that overexpressed cyr1-RAD bound and sequestered endogenous gpa2 from cyr1 and caused inhibition of the cAMP signaling pathway, thereby releasing its inhibitory effect on sporulation.

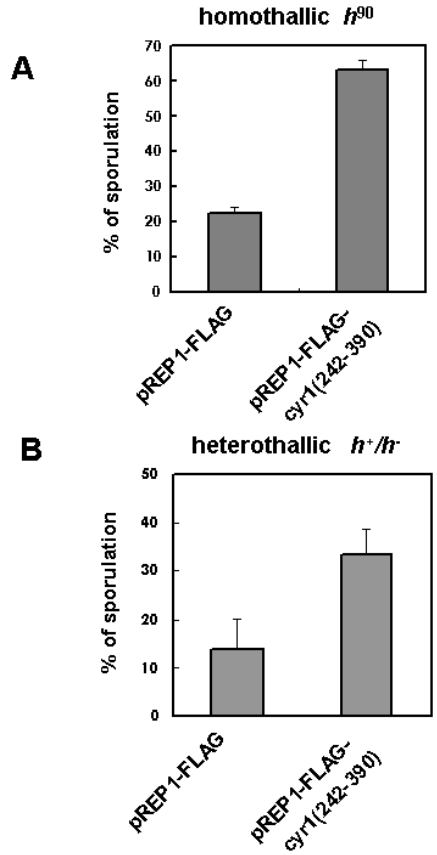

Fig. 3. Sexual development of yeast cells overexpressing cyr1-RAD. (A) Cells of homothallic strains SHO1 and SHO2 (Table I) were grown to the log phase in EMM medium, diluted to the density of 10^6 cells/ml, and inoculated onto SSA sporulation medium plates. Sporulation was observed under microscope after 2 days incubation at 30°C, and the percentage of asci was calculated. The values are expressed as the means ± S.D. (n = 4). (B) Cells of heterothallic strains HO1, HO2, HO3, and HO4 (Table I) were grown to the log phase and diluted to the density of 10^6 cells/ml. HO1 and HO2 were simultaneously inoculated on a SSA sporulation medium plate, and HO3 and HO4 were treated similarly. The sporulation efficiencies were determind as described in (A). The values are expressed as the means ± S.D. (n = 3).

In Vitro Activation of cyr1 by gpa2

We examined whether the observed direct association with gpa2 was sufficient to induce activation of cyr1. In order to reconstitute the gpa2-dependent activation of cyr1 in the membrane fraction, we used the posttranslationally modified form of gpa2 purified from the membrane fraction of yeast cells. The purified gpa2 showed two major bands, in which the upper band corresponded to FLAG-gpa2 (Fig. 4A). The identity of the lower band remained unknown even though it was likely to be git5, which was associated with gpa2. Because the purified gpa2 preparation showed a low GTP-loading efficiency, we used an alternative way of inducing gpa2 activation, that is, treating gpa2-GDP with AlF_4^-, which is known to be inserted into the position equivalent to the γ-phosphate of GTP (10, 36). The resulting gpa2-GDP-AlF_4^- complex was incubated with membrane fractions overexpressing cyr1 (Fig. 4B), and adenylyl cyclase activity was measured as described in MATERIALS AND METHODS (Fig. 4C). As shown in Fig. 3C, gpa2-GDP was incapable of activating cyr1 in

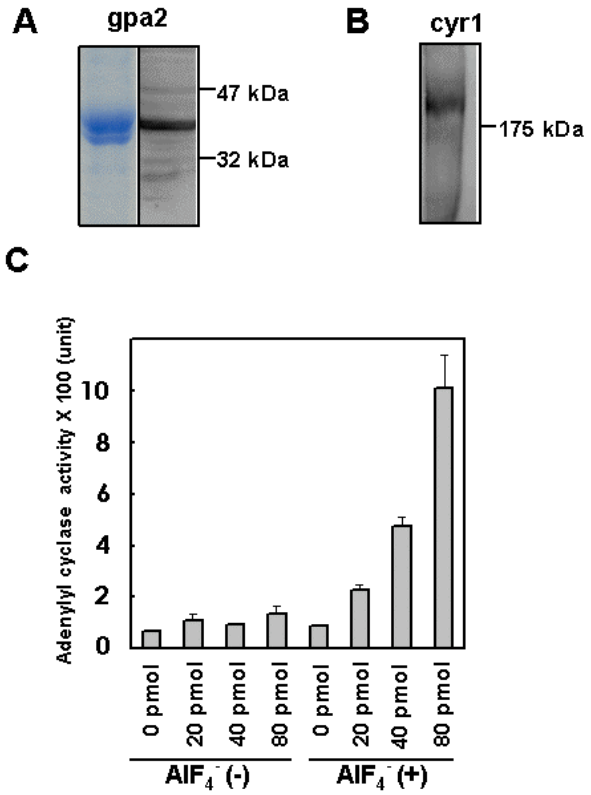

Fig. 4. Measurement of *in vitro* activation of cyr1 by gpa2. (A) FLAG-gpa2, purified from the membrane fraction of the yeast KN1, was fractionated by SDS polyacrylamide gel electrophoresis (10% gel) and detected by staining with Coomassie Brilliant Blue (left panel) or by immunoblotting with the anti-FLAG antibody (right panel). (B) FLAG-cyr1 in the membrane fraction of the yeast FS100 was subjected to immunodetection with the anti-FLAG antibody. (C) Adenylyl cyclase activities dependent on various concentrations of purified FLAG-gpa2 were measured in the presence or absence of AlF_4^- as described in MATERIALS AND METHODS. One unit of activity is defined as 1 pmol of cAMP formed in 1 min of incubation with 1 mg of membrane protein. The values are expressed as the means ± S.D. (n = 4).

the absence of AlF_4^-. In contrast, gpa2-GDP-AlF_4^- complex activated cyr1 in a dose-dependent manner. AlF_4^--treatment failed to induce cyr1 activation without gpa2. Taken together, these results indicated that gpa2 is capable of activating cyr1 through direct association at cyr1-RAD. Thus, RADs, which have been defined as a conserved motif shared among the Ras-family small G protein-associating domains, are for the first time shown to exhibit a functional association with a member of the heterotrimeric G proteins.

DISCUSSION

In this report, we have shown the first biochemical evidence that the GTP-bound active form of *S. pombe* gpa2 directly binds to cyr1 and induces its activation. The results are fully consistent with those obtained from a number of genetic studies on *cyr1* and *gpa2* reported so far. Thus, *S. pombe* share a common regulatory mechanism for adenylyl cyclase with mammals, that is, involvement of Gα subunits of heterotrimeric G protein. On the other hand,

the mechanism underlying the association of adenylyl cyclases with G proteins shares a common feature between *S. pombe* and *S. cerevisiae*, that is, involvement of RADs. Mammalian adenylyl cyclases do not possess any domain homologous to RAD, and their catalytic domains are shown to be responsible for direct association with Gα subunits (37). Molecular mechanisms underlying the gpa2-dependent cyr1 regulation remain to be clarified further. We presently do not know whether git5 and git11 are functional equivalents of mammalian Gβ and Gγ subunits, respectively, for gpa2. Because the cyr1 activity reconstituted *in vitro* appears to be much lower than RAS2-dependent activity of *S. cerevisiae* adenylyl cyclase, it may be possible that a certain critical component may be limiting in our reconstitution system.

RADs were discovered through a computer-based analysis as a motif conserved among the known Ras family small G protein-binding sites of various effector proteins, including Ral-GDS, Rin1, afadin/AF-6, phospholipase Cε and RA(PDZ)-GEF-1 and -2 (5, 21, 30, 35). This report provides the first biochemical evidence for the existence of its binding partner belonging to the heterotrimeric G protein family. RADs may also be involved in association with other heterotrimeric G proteins because a weak association between RAD-containing *S. cerevisiae* STE50 and GPA1 has been observed by using yeast two-hybrid system (31, 42). Further studies will be needed to clarify the molecular mechanisms whereby various G proteins specifically recognize their RAD counterparts.

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