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Gtr1p differentially associates with Gtr2p and Ego1p

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ABSTRACT

The yeast Ras-like small GTPases Gtr1p and Gtr2p form a heterodimer and interact genetically with Prp20p, a guanine nucleotide exchange factor for the GTPase Gsp1p. Gtr1p and Gtr2p may be involved in nucleocytoplasmic transport and in the nutrient-responsive TOR signaling pathway, but the role of the Gtr1p–Gtr2p heterodimer is not well understood. Characterization of the Gtr1p–Gtr2p complex is indispensable for understanding the functions of both Gtr1p and Gtr2p. We analyzed the association mode between Gtr1p and Gtr2p. The N-terminus nucleotide binding region of Gtr1p associated with Gtr2p, but not with Ego1p, a protein known to interact with Gtr1p. Gtr1p and Gtr2p are necessary for cells to acquire resistance to caffeine, rapamycin, and hydrogen peroxide. Caffeine treatment released Gtr1p from the high molecular weight Gtr1p–Gtr2p complex. Gtr2p mutants S23N and T44N, but not Q66L, rescued the *gtr2* disruptant. Our findings indicate that the formation of heterodimers by Gtr1p differs between Gtr2p and Ego1p.

1. Introduction

Growth factor-dependent or stress-induced signal transduction is mediated by the small GTPase Ras and its homologs (Campbell et al., 1998; Tzima, 2006). These proteins switch between an active GTP-bound form and an inactive GDP-bound form and act as molecular switches for various signaling pathways (Bourne et al., 1990).

Ras-like small GTPases in yeast comprise Ras1p, Ypt1p, Cdc42p, Arf1p, Gtr1p, and Gsp1p. Among these, Gtr1p is unique because it can form a homodimer as well as a heterodimer with Gtr2p, another member of the Gtr1p subfamily of Ras-like small GTPases (Nakashima et al., 1999), whereas other small GTPases are strictly monomeric (Sprang, 1997). Gtr1p negatively regulates the Ran/Gsp1p cycle through Gtr2p (Nakashima et al., 1999). Gtr1p interacts either genetically or biochemically with nuclear and cytoplasmic proteins, such as Rpc19p, Nop8p, Yrb2p (Taura et al., 1998; Sekiguchi et al., 2004; Todaka et al., 2005; Wang et al., 2005), and Ego1p and Ego3p (Huang et al., 2004). In this report, we examined the significance of the Gtr1p–Gtr2p dimer and Gtr2p mutants.

2. Materials and methods

2.1. Strains, media, and two-hybrid assay

The *Saccharomyces cerevisiae* strains used in this study are listed in Supplementary Table 1. XGY53 was kindly provided by Dr. Neta Dean (Gao et al., 2005). Yeast strains were grown as previously described (Wang et al., 2005). *S. cerevisiae* was transformed using the lithium-acetate method with DMSO (Hill et al., 1991). Yeast two-hybrid assays (Chien et al., 1991) were performed as described previously (Sekiguchi et al., 2001). Caffeine (Nacalai Tesque, Kyoto, Japan) was used at a final concentration of 1.5 mg/ml. Rapamycin (Nacalai Tesque) was used at a concentration of 7.5 or 10 ng/ml.

2.2. Plasmid construction

The plasmids used in this study are listed in Supplementary Table 2. Each construct was validated by sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Abbreviations: TOR, target of rapamycin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GTP, Guanosine Triphosphate; GDP, Guanosine Diphosphate; DBD, DNA binding domain; AD, Activation Domain; HA, hemagglutinin; Mr, Molecular weight; PDB, Protein Data Bank.

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2.3. Immunoprecipitation, immunoblotting, and antibodies

Yeast protein extracts for SDS-PAGE and immunoblotting were prepared as described previously (Adams et al., 1997). Protein samples were electrophoresed on a 5% to 20% gradient gel (PAGEL, Atto Corporation, Japan) and 10% polyacrylamide gel and analyzed by immunoblotting as described previously (Sekiguchi et al., 2001) using an ECL kit (GE Healthcare). Mouse anti-hemagglutinin (HA) antibody (Cat. no. MMS-101P) was purchased from CRP Inc. Mouse anti-MYC (9E10) and rabbit anti-GST (Z-5) antibodies were purchased from Santa Cruz Biotechnology.

2.4. Gel filtration chromatography

Yeast proteins were size-fractionated as described previously (Ebbert et al., 1999; Wang et al., 2004). A complex of GST-Gtr1p and T7-His-Gtr2p (100 pmol) was preloaded with GTP or GDP and suspended in lysis buffer (Wang et al., 2005). The mixtures were then subjected to gel filtration using a HiLoad 16/60 Superdex 200-pg (GE Healthcare).

2.5. Nucleotide-binding assay

The Gtr1p–Gtr2p complex and Gtr2p were purified as described previously (Wang et al., 2005). The binding of these proteins with [^{3}H] GDP or ^{35}S-labeled GTPγS was performed as described previously (Noguchi et al., 1997; Wang et al., 2005).

3. Results

3.1. Gtr1p–Gtr2p binding regions

We used a yeast two-hybrid assay to examine the region(s) in Gtr1p that bind to Gtr2p. Gtr1p residues 1 to 233 (effector-binding region) bound to Gtr2p (Fig. 1A), but only the deletion of the N-terminal 20 residues of Gtr1p (nucleotide-binding region) disrupted the association. Gtr1p (1–200) did not bind to Gtr2p, but Gtr1p (1–233) bound to Gtr2p, so it is likely that Gtr1p (200–233) binds to Gtr2p or that the coordination between the N-terminus (1–20) and the middle region (200–233) is important for this interaction due to the tertiary structures of the proteins (Fig. 1A).

We next determined the region in Gtr2p that binds Gtr1p. Serial deletion clones of *GTR2* in pACT2 were constructed and used in a two-hybrid analysis with a β-galactosidase assay to evaluate the interaction (Fig. 1B). The 50–341 Gtr2p construct, which lacks a part of the nucleotide- and effector-binding switch I regions, interacted with Gtr1p. The 50–341 Gtr2p construct did not rescue the *GTR2* disruptant.

We next investigated the stability of the Gtr1p–Gtr2p complex (assembled from purified Gtr1p and Gtr2p) in the presence of GTP or GDP in vitro. Preloaded GTP in the Gtr1p–Gtr2p complex was not exchanged for [^{3}H]GDP or [^{3}H]GTP, although preloaded GDP was readily exchanged for [^{3}H]GTP but not [^{3}H]GDP (Supplementary Fig. 1A). Gel filtration analysis of the Gtr1p–Gtr2p complex in the presence of either GTP or GDP (Supplementary Fig. 1B) showed that Gtr1p–Gtr2p binding to GTP facilitated the formation of a high molecular weight complex in vitro.

Therefore, the C-terminal region of Gtr1p (234–310) was not required for the interaction with Gtr2p (Fig. 1A), suggesting that these residues are required for interactions with other proteins. To test this idea, we constructed various Gtr1p deletion clones and examined their interactions with known interacting proteins. The *gtr1 150–310* interacted with Ego1p (Fig. 1C), demonstrating that residues in the Gtr1p N-terminal nucleotide binding region are not required for the interaction with Ego1p, and suggesting that Ego1p is not a Gtr1p effector protein. Gtr1p and Gtr2p belong to the same subfamily of Ras-like small GTPases and have high sequence similarity, therefore their three-dimensional structures might be similar. Thus, we used the three-dimensional structure of RagD/RRAG D (PDB; 2Q3F; human Gtr2p) to model the Ego1p-associating region of Gtr1p (Fig. 1C, red), which is located on the opposite side of Gtr1p relative to the switch I and II regions (Fig. 1C, yellow) to which Gtr2p and possible effectors bind. Because the *gtr2S23L* mutant had similar biologic activity as wild-type *GTR2* (see below), we used *gtr2S23L* as a bait in a yeast two-hybrid system to show that Gtr2p interacted with Ego1p (Fig. 1D). Wild-type *GTR2* was not used because it activates transcription in the two-hybrid system (Sekiguchi et al., 2008). Gtr2p deletion clones did not interact with Ego1p (Fig. 1D). Given that no Gtrp2 deletion mutants interacted with Ego1p, we propose that Gtr2p also interacts with Ego1p independent of Gtr1p. It remains to be determined, however, whether these three proteins exist in one complex or in different complexes. Based on the current data, we conclude that Gtr1p interacts with Gtr2p at the N-terminus to 233 a.a. and with Ego1 at the C-terminus.

To further understand the significance of the Gtr1p–Ego1p interaction, we performed gel filtration analysis of cell lysates expressing either Gtr1p, Gtr1 1–200p, or Gtr1 150–310p and Gtr2p, Gtr2 1–200p, or Gtr2 150–341p (Fig. 1E). These polypeptides were expressed from *GTR1* or *GTR2* constructs fused with the *GAL4* DNA binding domain (DBD) or GAL4 activation region (AD) in the Y190 yeast strain. GAL4DBD-Gtr1p and GAL4AD-Gtr2p have biologic activity (Sekiguchi et al., 2008) (Fig. 1B). GAL4DBD-Gtr1 150–310p, which associated with Ego1p (Fig. 1C), was present mainly in the higher molecular weight fractions 21–27, whereas a substantial portion of GAL4DBD-Gtr1 1–200p, which did not associate with Ego1p, was present in the lower molecular weight fractions 29–41. In the *EGO1* disruptant, GAL4 DBD-Gtr1p was present in the higher molecular weight fractions (Fig. 1E), indicating that Ego1p is not required for Gtr1p assembly into higher molecular weight complexes. Similarly, a substantial portion of GAL4 AD-Gtr2 1–200p was present in the lower molecular weight fractions 29–41, whereas GAL4AD/wild-type Gtr2 and 150–341p were present in high molecular weight fractions. In the *EGO1* disruptant, GAL4 AD-Gtr2p was present in the higher molecular weight fractions, indicating that Ego1p is not required for Gtr2p assembly into higher molecular weight complexes.

3.2. Effect of TOR pathway inhibition on Gtr1p–Gtr2p dimerization

Gtr1p and Gtr2p are involved in the heat shock response in yeast (Nakashima et al., 1996, 1999). Gtr1p and Gtr2p are also involved in pathways that are activated during nitrogen starvation and rapamycin treatment, suggesting that they have roles in the TOR kinase pathway (Dubouloz et al., 2005; Gao and Kaiser, 2006). Δ*gtr1* and Δ*gtr2* strains had a similar rapamycin- and caffeine-sensitive phenotype (Fig. 2A, upper panels), which is consistent with recent reports that caffeine inhibits TOR kinase activity (Kuranda et al., 2006; Reinke et al., 2006). Mammalian TOR regulates certain mitochondrial functions, including oxidation (Schieke et al., 2006). Consistent with these observations, hydrogen peroxide induced an oxidative response and inhibited cell growth in Δ*gtr1*, Δ*gtr2*, and Δ*gtr1*Δ*gtr2* strains (Fig. 2A, lower panels).

The levels of many stress response proteins, such as heat shock proteins, increase during exposure to stressors (Lindquist, 1986). Thus, to elucidate how Gtr1p and Gtr2p respond to a stressful stimulus we examined changes in the levels of these proteins after adding caffeine. We constructed a yeast strain of Δ*gtr1*Δ*gtr2* expressing MYC-tagged Gtr2p and HA-tagged Gtr1p driven by their own promoters. The overnight culture medium was changed to stimulate cell proliferation (time 0; Fig. 2B, lane 1). Both Gtr1p and Gtr2p levels increased after growth stimulation (Fig. 2B, lanes 2, 3). This increase in Gtr1p and Gtr2p levels was blocked within 1 h by the addition of caffeine (Fig. 2B,