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Yeast Fps1 Glycerol Facilitator Functions as a Homotetramer

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Abstract

The *S. cerevisiae* Fps1 glycerol channel is a member of the major intrinsic protein (MIP) family of plasma membrane channel proteins that functions in osmoregulatory pathways to transport glycerol passively out of the cell. The MIP family is subdivided into members that are selectively permeable to water (aquaporins), and those permeated by glycerol (aquaglyceroporins or glycerol facilitators). Although aquaporins function as homo-tetramers with each monomer possessing its own channel, previous studies have suggested that aquaglyceroporins may function as monomers. Here we provide both genetic and biochemical evidence that Fps1 functions as a homotetramer to regulate glycerol transport in yeast.

INTRODUCTION

Under conditions of high osmolarity stress, many fungal species, including *S. cerevisiae*, maintain osmotic equilibrium by producing and retaining high concentrations of glycerol as a compatible solute (Nevoigt and Stahl, 1997). Intracellular glycerol concentration is regulated in *S. cerevisiae* in part by the Fps1 plasma membrane glycerol channel (Luyten et al., 1995; Sutherland et al., 1997; Tamas et al., 1999). Increased external osmolarity induces Fps1 closure, whereas decreased osmolarity causes channel opening, both within seconds of the change in external osmolarity (Tamas et al., 1999). This channel is required for survival of a hypo-osmotic shock when yeast cells have to export glycerol rapidly to prevent bursting (Luyten et al., 1995; Tamas et al., 1999) and is required for controlling turgor pressure during fusion of mating yeast cells (Philips and Herskowitz, 1997).

Fps1 is a member of the major intrinsic protein (MIP) family of channel proteins. The MIP family is subdivided into members that are selectively permeable to water (aquaporins) and those permeated by glycerol and to a lesser extent by water, called aquaglyceroporins, or glycerol facilitators (Borgnia and Agre, 2001; Agre et al., 2002). Numerous aquaporins have been shown to function as homotetramers, with each monomer possessing its own channel (Aerts et al., 1990; Smith and Agre, 1991; Verbavatz et al., 1993; Shi et al., 1994; Beuron et al., 1995; Konig et al., 1997). Additionally, a three-dimensional structure has been determined for the human aquaporin AQP1 tetramer (Watz et al., 1997; Murata et al., 2000; Sui et al., 2001; de Groot et al., 2003). In contrast to this, the only aquaglyceroporin characterized with regard to subunit structure is the *E. coli* GlpF protein, which sediments as a monomer through sucrose density gradients (Lagréé et al., 1998; Duchesne et al., 2001). However, GlpF appears variously as monomers or tetramers in membranes examined by freeze-fracture (Bron et al., 1999) or cryo-electron microscopy (Braun et al., 2000), and its crystal structure reveals a tetrameric complex (Fu et al., 2000). Based on the results of

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detailed velocity sedimentation experiments, Borgnia and Agre (2001) concluded that GlpF exists in multiple oligomeric states, ranging from monomers to tetramers depending upon salt concentration. These findings are in contrast to the high stability of aquaporin tetramers and underscore the question of which form is functional.

Sequence comparisons among MIP family members have led to the identification of five key residues that distinguish between aquaporins and aquaglyceroporins (Froger et al., 1998). Among these is a pair of adjacent residues within transmembrane helix 6 that are highly conserved as Tyr/Phe-Trp in aquaporins, but not in the glycerol facilitators. Substitution of these residues with Pro and Leu, respectively, in an aquaporin from *Cicadella viridis* (AQP_{cic}) to mimic *E. coli* GlpF, converted this tetrameric water channel to a monomeric glycerol facilitator (Lagrée et al., 1999). This finding supported the suggestion that the quaternary structure of MIP family members plays an important role in defining their specificity and that glycerol facilitators, as a group, function as monomers (Lagrée et al., 1998). Here we present genetic and biochemical evidence that the yeast Fps1 glycerol facilitator self-associates in its functional state.

MATERIALS AND METHODS

Strains and growth conditions

All experiments were carried out in the Research Genetics S288c (BY4742) background *MATa his3Δ leu2Δ ura3Δ lys2Δ*. Haploid strains were DL3187 (BY4742) and DL3226 (*fps1Δ::KanMX*). Diploid strains, DL3209 (*MATa/MATa rgc1Δ::KanMX rgc2Δ::KanMX*) and DL3245 (*MATa/MATa rgc1Δ::KanMX rgc2Δ::KanMX fps1Δ::KanMX*), were described previously (Beese et al., 2009). Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose) or SD (0.67% Yeast nitrogen base, 2% glucose) supplemented with the appropriate nutrients to select for plasmids. Intracellular glycerol concentrations were measured as described previously (Beese et al., 2009).

FPS1 plasmids

Episomal plasmids pRS202 [*FPS1*] (p2165) and pRS426 [*MET25-FPS1-Flag*] (p2492), and centromeric plasmid pRS316 [*FPS1*] (p2833) were described previously (Beese et al., 2009). The *fps1-Δ1* allele was subcloned from YEpl95 [*fps1-Δ1*] (p2496) into centromeric plasmid, pRS316, to yield pRS316 [*fps1-Δ1*] (p2834). *FPS1-Myc* was placed under the control of the *MET25* promoter by PCR amplification of the promoter and most of the coding region from pRS426 [*MET25-FPS1-Flag*] (p2492), followed by subcloning of this fragment into the SalI-ApaI sites of YEpl81 [*FPS1-Myc*] (p2184; Beese et al., 2009), which replaced the endogenous promoter with *MET25* to generate YEpl81 [*MET25-FPS1-Myc*] (p3121). Primers were: *MET25* SalI Forward: GATCGTCGACAGCTCCGGATGCAAGGGTTC, and *FPS1* 1824 reverse: TCCTGCTTCATTTTCGTTGTC. This construct was validated by DNA sequence analysis.

Co-immunoprecipitation and crosslinking

Cultures grown to mid-log phase in selective medium for co-immunoprecipitation experiments were starved for methionine for 2 hours to induce expression of Fps1-Flag and Fps1-Myc. Protein extraction and co-immunoprecipitation were carried out as described previously (Kamada et al., 1995), except that 0.5% Triton X-100 was included in the lysis buffer. Extracts were exposed to mouse monoclonal α-FLAG antibody (M2; Sigma) for 1 hour and precipitated with protein A affinity beads (Sigma) for 1 hour at 4°C. Samples were separated by SDS-PAGE (7.5% gels) followed by immunoblot analysis using M2 antibody, or α-Myc antibody (9E10; BabCo) at a dilution of 1:10,000. Secondary antibodies (goat anti-mouse; Amersham) were used at a dilution of 1:10,000. For dimethyl suberimidate

(DMS) crosslinking, Fps1-Flag immunoprecipitates from extracts (extraction buffer: 50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 5mM EGTA, 0.2mM Na₃VO₄, 50mM KF, 30mM Na₂PPi, 0.02mg/mL leupeptin and benzamidine, 0.01mg/ml pepstatinA, 0.04mg/ml aprotinin, PMSF, 1mM DTT, 0.5% NP-40, 20% glycerol) were treated with various concentrations of DMS in 50mM HEPES buffer (pH 7.0) for 2 hours at room temperature prior to separation of proteins by SDS-PAGE.

RESULTS AND DISCUSSION

We showed previously that the Rgc1 and Rgc2 proteins are positive regulators of the Fps1 glycerol facilitator (Beese et al., 2009). An *rgc1/2Δ* mutant fails to grow at 37°C because it undergoes cell lysis due to the elevated turgor pressure associated with glycerol hyper-accumulation. This phenotype was shown to be the consequence of a deficiency in the function of the Fps1 glycerol facilitator, rather than to a problem with either its expression or localization. Fps1 channels retain a low level of activity (less than 5% of normal) in an *rgc1/2Δ* mutant, and the *rgc1/2Δ* growth defect is suppressed by over-expression of Fps1 from a 2μ plasmid (Beese et al., 2009).

In the course of our studies to understand how Rgc1 and Rgc2 regulate Fps1, we examined the ability of a constitutively open channel mutant form of Fps1 (Fps1-Δ1) to suppress the *rgc1/2Δ* growth defect. The *fps1-Δ1* mutant is unable to accumulate glycerol in response to hyper-osmotic shock because the encoded protein is missing most of the N-terminal cytoplasmic extension (residues 12–231), which is thought to act as a regulatory domain that closes the channel (Tamas et al., 1999). Therefore, we anticipated that low-level expression of this constitutive Fps1 form would suppress the *rgc1/2Δ* growth defect. Contrary to expectation, expression of *fps1-Δ1* from a centromeric plasmid was only able to suppress the *rgc1/2Δ* growth defect in the absence of wild-type *FPS1* (Figure 1), revealing that the constitutive mutation is recessive to the wild-type allele with respect to this phenotype. This indicated that the constitutive Fps1-Δ1 form takes on its open channel character only in the absence of wild-type Fps1 protein, and suggested that Fps1 monomers function together in a multimeric complex. Indeed, previous studies of Fps1 open channel forms have all employed their over-expression from 2μ plasmids, and typically in the absence of wild-type Fps1 (Tamas et al., 1999; 2003; Hedfalk et al., 2004; Beese et al., 2009).

To test more directly the open channel character of the Fps1-Δ1 form in the presence or absence of wild-type Fps1, we next measured glycerol accumulation in response to hyper-osmotic stress. Expression of Fps1-Δ1 in yeast cells also expressing the endogenous wild-type Fps1, which closes in response to hyper-osmotic shock, had little effect on their ability to accumulate glycerol after shift to 1.8 M sorbitol (Figure 2). However, in an *fps1Δ* mutant, which hyper-accumulates glycerol even in the absence of hyper-osmotic stress, *fps1-Δ1* greatly diminished glycerol accumulation and was not responsive to hyper-osmotic stress. This result supports the conclusion that the *fps1-Δ1* allele is recessive to wild-type *FPS1*. We noted additionally that the basal level of glycerol retention in the *fps1-Δ1* mutant in an *fps1Δ* background was somewhat higher than that of the wild-type strain, suggesting that the unregulated channel activity of this form is somewhat lower than the basal activity of wild-type Fps1. However, Luyten et al. (1995) observed that over-expression of wild-type Fps1 inexplicably resulted in elevated glycerol production and retention. Therefore, it is possible that the *fps1-Δ1* mutant has elevated channel activity that is not reflected by the retention assay because of a compensatory feedback effect on glycerol production.

Genetic analyses indicating that the *fps1-Δ1* allele is recessive to wild-type *FPS1* suggested the possibility that Fps1 functions as a multimeric complex. Therefore, we tested the ability of Fps1 to self-associate *in vivo* by co-immunoprecipitation of differentially epitope-tagged

forms of the glycerol channel. Figure 3 shows that Myc-tagged Fps1 co-precipitates with immunoprecipitated Flag-tagged Fps1, indicating that Fps1 self-associates in a multimeric complex. To determine the nature of the Fps1 complex, we carried out crosslinking experiments using Flag-tagged Fps1 immunoprecipitated from wild-type cells. Immunoblots of immunoprecipitated Fps1 treated with increasing concentrations of the crosslinking agent, dimethyl-suberimidate (DMS; Davies and Stark, 1970), revealed a ladder of bands corresponding approximately in molecular weight to one, two, three, and four molecules of Fps1-Flag (monomeric molecular weight, 75kDa; Figure 4). No additional bands were detected that migrated more slowly than the presumptive Fps1 tetramer even upon overexposure of the film.

Our data reveal that the likely explanation for the recessive nature of the *fps1-ΔI* mutation is that the encoded glycerol channel functions in homo-multimeric form, most likely as a tetramer. These findings stand in contrast to the conclusion of Lagrée et al. (1998, 1999) that glycerol facilitators function as monomers, whereas aquaporins are tetramers. A mutant form of the AQPcic aquaporin (Y222P, W223L) that exists as a monomer and transports glycerol not only disrupts the quaternary structure of the tetrameric complex, but also changes its specificity (Lagrée et al., 1999). This correlative observation supported an earlier suggestion by the same group that quaternary structure in some way influences channel specificity (Lagrée et al., 1998). The mutation site resides within transmembrane helix 6 which, based on the crystal structures of AQP1 and GlpF, is involved in contacts between monomers (Walz et al., 1997; Fu et al., 2000), but does not participate directly in pore formation. Therefore, it is possible that this mutation only coincidentally impacts pore specificity of AQPcic. In any event, our results with Fps1 fail to support the suggestion that all glycerol facilitators act as monomers.

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Figure 1.

Low copy expression of the *fps1Δ-1* constitutive open channel allele suppresses the cell lysis defect of an *rgc1/2Δ* mutant only in the absence of wild-type *FPS1*. A diploid *rgc1/2Δ* double mutant (DL3209) and *rgc1/2Δ fps1Δ* triple mutant (DL3245) were transformed with a low-copy *FPS1* plasmid (pRS316 [*FPS1*]; p2833), a high-copy *FPS1* plasmid (pRS202 [*FPS1*]; p2165), a low-copy *fps1-Δ1* plasmid (pRS316 [*fps1-Δ1*]; p2834), or vector (pRS316). Transformants were streaked onto selective plates at 37°C for 4 days.

Figure 2.

The *fps1-Δ1* constitutive open channel allele is recessive to *FPS1* for glycerol accumulation in response to hyper-osmotic shock. Wild-type (DL3187) and *fps1Δ* (DL3226) strains transformed with the indicated plasmid were grown to mid-log phase in YPD and diluted into YPD with or without sorbitol (to 1.8M) to induce hyper-osmotic shock (15 minutes). Cells were collected and intracellular glycerol concentrations were determined. Each value represents the mean and standard deviation from three independent experiments.

Figure 3.

Self-association of Fps1. Wild-type yeast (DL3187) was co-transformed with plasmids pRS426 [*MET25-FPS1-Flag*] (p2492) and YEp181 [*MET25-FPS1-Myc*] (p3121), or empty vectors for co-expression of Fps1-Flag and Fps1-Myc. Fps1-Flag was immunoprecipitated (IP) from extracts (Input) and subjected to SDS-PAGE separation and immunoblot (IB) analysis for detection of Fps1-Flag and co-precipitating Fps1-Myc.

Figure 4.

Fps1 exists as a homo-tetramer *in vivo*. Fps1-Flag was immunoprecipitated from wild-type yeast (DL3187) transformed with pRS425 [*MET25-FPS1-Flag*] (p3042) and immunoprecipitates were treated with the indicated concentration of DMS to introduce inter-subunit crosslinks. Samples were subjected to SDS-PAGE separation and immunoblot analysis. The panel on the right shows the same samples as the panel on the left, but subjected to longer electrophoretic separation.