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A Regulatory Domain in the C-terminal Extension of the Yeast Glycerol Channel Fps1p*

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The *Saccharomyces cerevisiae* gene *FPS1* encodes an aquaglyceroporin of the major intrinsic protein (MIP) family. The main function of Fps1p seems to be the efflux of glycerol in the adaptation of the yeast cell to lower external osmolarity. Fps1p is an atypical member of the family, because the protein is much larger (669 amino acids) than most MIPs due to long hydrophilic extensions in both termini. We have shown previously that a short domain in the N-terminal extension of the protein is required for restricting glycerol transport through the channel (Tamás, M. J., Karlgren, S., Bill, R. M., Hedfalk, K., Allegri, L., Ferreira, M., Thevelein, J. M., Rydström, J., Mullins, J. G. L., and Hohmann, S. (2003) *J. Biol. Chem.* 278, 6337–6345). Deletion of the N-terminal domain results in an unregulated channel, loss of glycerol, and osmosensitivity. In this work we have investigated the role of the Fps1p C terminus (139 amino acids). A set of eight truncations has been constructed and tested *in vivo* in a yeast *fps1*Δ strain. We have performed growth tests, membrane localization following cell fractionation, and glycerol accumulation measurements as well as an investigation of the osmotic stress response. Our results show that the C-terminal extension is also involved in restricting transport through Fps1p. We have identified a sequence of 12 amino acids, residues 535–546, close to the sixth transmembrane domain. This element seems to be important for controlling Fps1p function. Similar to the N-terminal domain, the C-terminal domain is amphiphilic and has a potential to dip into the membrane.

Aquaporins and aquaglyceroporins comprise a large family (also called the MIP[1] family) of integral membrane proteins in bacteria, fungi, plants, and animals. MIP channels mediate transport of water and small neutral solutes and/or ions (1); hence, they are specific for osmotic regulation at different levels. The importance of properly functional and regulated aquaporins is illustrated by their suspected involvement in numerous disorders such as congestive heart failure, glaucoma, and brain edema (2). MIPs share a common topology with six transmembrane helical segments per subunit (3, 4). The two halves of the proteins show similarity to each other indicative of gene duplication during the evolution of MIP channels (4). Two NPA (Asn-Pro-Ala) boxes, located in loops B and E respectively, are highly conserved, indicating that these residues have major importance for structure and/or function (5). Based on high-resolution structures of bovine AQP1 and *Escherichia coli* GlpF, the NPA motifs were shown to form a crucial part of the channel (4, 6–8). Most MIPs are <300 aa long, but there are examples of much larger proteins in the family, mainly of eukaryotic origin. The larger size is primarily due to extended N- and C-terminal domains, the poor conservation in these domains suggesting their involvement in regulation rather than function. This is exemplified by *Drosophila* Bib and Fps1p, which have distinct N- and C-terminal extensions that are homologous neither to each other nor to other proteins in the data base.

The strategy for accumulating compatible solutes in osmoadaptation is evolutionarily well conserved in bacteria, archaea, and eukarya, although the solutes differ (9, 10). A compatible solute increases the internal osmolarity and keeps proteins hydrated, leading to stabilization and protection of enzymes. Glycerol is the main compatible solute in growing *Saccharomyces cerevisiae* cells. The production of glycerol is stimulated under hyper-osmotic stress, and accumulated glycerol is released when the external osmolarity drops (11). Fps1p is a glycerol facilitator that controls the cytoplasmic concentration of glycerol (12). Mutants lacking Fps1p accumulate more intracellular glycerol, indicating that Fps1p is involved in glycerol efflux (12). This is further supported by the observation that *fps1*Δ mutants are sensitive to a hypo-osmotic shock and unable to export glycerol (13). Glycerol influx via Fps1p has also been observed (12). However, because mutants lacking Fps1p do not show a growth defect on glycerol as the sole carbon source, the physiological role of Fps1p appears to be efflux rather than uptake. This indicates the presence of other systems for glycerol uptake (13, 14).

Fps1p consists of 669 amino acids and is localized to the plasma membrane of *S. cerevisiae* (13). The protein has ~30% identity to GlpF within the transmembrane core (between amino acids 250 and 530) (15) but is one of the most divergent members of the MIP family. For instance, the two well con-

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[1] The abbreviations used are: MIP, major intrinsic protein; PBS, phosphate-buffered saline; TMD, transmembrane domain; aa, amino acid.

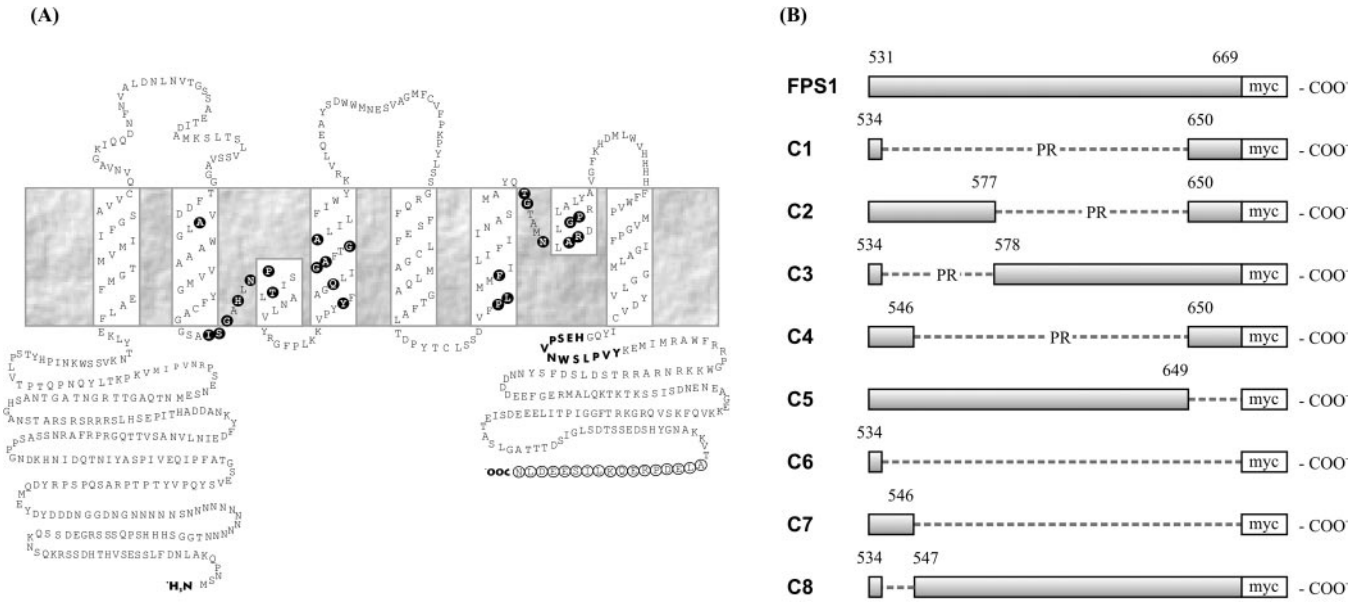

FIG. 1. **Topology model of Fps1p and the constructs used in this study.** *A*, conserved residues in the MIP family (●) and the C-terminal myc tag (○), used for detection on Western blot, are *highlighted*. *Bold font* corresponds to C-terminal residues found in this study to be important for proper channel regulation. *B*, the positions of each truncation in the C-terminal constructs, C1–C8, used in this study. The numbers refer to the last and the first amino acid before and after the truncation, respectively. All truncations have a C-terminal myc tag to enable detection on a Western blot. C1–C4 have a proline-arginine (*PR*) duplet initially introduced from the vector PCR to allow religation of the vector following a restriction digest. The latter constructs were made by gap repair, and the PR duplet was then excluded.

served NPA motifs in loops B and E are NPS and NLA in Fps1p (16) (Fig. 1*A*). Deletion of the hydrophilic N-terminal domain results in an unregulated channel, which, in turn, causes sensitivity to high osmolarity and glycerol overproduction (13). Further investigations have shown that a domain of 12 amino acids close to the first transmembrane domain is crucial for controlling Fps1p function. Deletion or specific point mutations in this domain render the channel unregulated (17). In this study we have found that the Fps1p C terminus has a similar regulatory role in the channel's function. When deleted, cells become sensitive to hyper-osmotic conditions, indicating a role for the C terminus in restricting transport. Moreover, a domain of 12 residues next to the last transmembrane domain has been identified as important for proper regulation. Notably, sequence similarities are found between the important domains in each terminus, as well as a potential to dip into the hydrophobic interior of the membrane.

EXPERIMENTAL PROCEDURES

Yeast Strain and Growth Conditions—The yeast strain used in this study is W303-1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1can1-100 GAL SUC2 mal0*) (18). Deletion of the *FPS1* gene or the *GPD1* and *GPD2* genes results in the strains *fps1*Δ::*HIS3* (YMT2) (17) and *gpd1*Δ::*TRP1 gpd2* Δ::*URA3* (19), respectively. Yeast cells were routinely grown in medium containing 2% peptone and 1% yeast extract supplemented with 2% glucose as carbon source (YPD). Selection and growth of transformants carrying a replicating plasmid was performed in yeast nitrogen base medium (YNB) (20) selecting for the *LEU2* marker.

Plasmid Constructions—The truncations were all based on the 2μ*LEU2* plasmid YEp181*myc-FPS1*, in which the c-*myc* epitope is attached to the carboxyl terminus of Fps1p (13). C1 and C2 were made from a vector PCR using the Expand Long Template PCR system (Roche Applied Science). A SacII restriction site was introduced to allow linearization and religation. The ApaI-XbaI fragment, containing the truncated part, was subcloned into the original vector, and the region of interest was sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Plasmid C3 was constructed as described above, with the exception that the PCR fragment was combined with the two annealed primers containing the truncation. The two DNA pieces were combined by gap repair (21) after cotransformation into yeast. The truncated part was subcloned and sequenced as described previously. C4–C7 were all made by gap repair; C4 on C2 linearized with SacII, and C5 on YEp181*myc-FPS1* linearized with XbaI, as did C6 and C7 on C4. The truncated part of C4–C7 was sequenced as described above. C8 was made by a vector PCR, where the template was digested by DpnI before transformation of the PCR product to *E. coli*. The truncated part was subcloned and sequenced as described previously. See Table I for a list of primers and primer sequences for the constructs.

Growth Assay—For growth assays, cells of the *fps1*Δ mutant were transformed with an empty plasmid or a plasmid containing the wild type gene or the C-terminal truncations. For a qualitative plate growth assay, cells were pregrown on YNB plates supplemented with 2% glucose and resuspended in the same medium to an OD_{600} of 0.3. 10 μl of the cell suspension, and three serial 1:10 dilutions were spotted on medium without sorbitol as a control and with 1 M sorbitol for a hyper-osmotic shock. For a hypo-osmotic shock, cells were pregrown on YNB plates supplemented with 2% glucose containing 1 M sorbitol and spotted on plates with sorbitol as control and without sorbitol for the hypo-osmotic shock. Plates were incubated at 30 °C, and growth was monitored after 2–3 days. For the more quantitative growth assay, cells were pregrown in YNB supplemented with 2% glucose for 16 h and diluted to an OD_{600} of 0.15 in the same medium. For hyper-osmotic conditions, cells from the preculture were collected by centrifugation at 3000 × *g* for 10 min at room temperature and resuspended in YNB and 1.5 M sorbitol supplemented with 2% glucose and with OD_{600} adjusted to 0.15. 350-μl cultures were assayed in a LabSystems Bioscreen C for 4–6 days. The observed OD values have been transformed to OD values that are corrected for non-linearity (22). The average generation time, yield, and growth curve were calculated for three transformants from each construct. To test for the growth advantage of an unrestricted Fps1p channel on xylitol, plasmids were transformed into a *gpd1*Δ *gpd2*Δ strain, which is defective in glycerol production (19).[2] Cells were pregrown on YNB plates supplemented with 2% glucose and resuspended in the same medium to an OD_{600} of 0.5. Five microliters of the cell suspension and three serial 1:10 dilutions were spotted on YNB plates supplemented with 2% glucose without and with 1 M xylitol, respectively.

Membrane Preparation and Immunoblots—For membrane preparations, cells were grown in YNB supplemented with 2% glucose to an OD_{600} of 0.5–0.7, *i.e.* mid-log phase. The total membrane fraction was prepared as described previously (13). Protein concentration was measured with a Bio-Rad DC protein assay using bovine serum albumin as standard. 10–400 μg of total protein was loaded in each lane and separated by SDS-PAGE. Proteins were then transferred to a polyvi-

[2] S. Karlgren, N. Petersson, R. M. Bill, and S. Hohmann, unpublished observations.

TABLE I
Oligonucleotides used for constructing the set of eight C-terminal truncations of FPS1

Construct	Primer	Primer sequence[a]
C1, C2	$FPS1_{1948–1965}$	5′-tccccgcggATTGGGTTATCCGACACA-3′
C1	$FPS1_{1602–1585}$	5′-tccccgcggACCCTGATAAATACAGAC-3′
C2	$FPS1_{1732–1714}$	5′-tccccgcggGTATGAGAAGTCACTCAG-3′
C3	$FPS1_{1717–1750}$	5′-ggcacatacccgcggAATAACGATGATGATGAGG-3′
C3	$FPS1_{1617–1581}$	5′-catacacggccgcggACCCTGATAAATACAGACATCG-3′
C3	C3.GAP.S	5′-TACGATGTCTGTATTTATCAGGGTccgcggAATAACGATGATGATGAGGAATTTG-3′
C3	C3.GAP.AS	5′-CAAATTCCTCATCATCATCGTTATTccgcggACCCTGATAAATACAGACATCGTA-3′
C4	C4.GAP.S	5′-AGTCAACTGGTCTTTACCAGTTTATccgcggATTGGGTTATCCGACACATCATCAG-3′
C4	C4.GAP.AS	5′-CTGATGATGTGTCGGATAACCCAATccgcggATAAACTGGTAAAGACCAGTTGACT-3′
C5	C5.GAP.S	5′-TTCGCTAGGTGCGACGACGACTGATTCTGCTCTAGAGGATCCCCGGGAACAAAAACTTATTTCTGA-3′
C5	C5.GAP.AS	5′-TCAGAAATAAGTTTTTGTTCCCGGGGATCCTCTAGAGCAGAATCAGTCGTCGTCGCACCTAGCGAA-3′
C6	C6.GAP.S	5′-GGTTTACGATGTCTGTATTTATCAGGGTGCTCTAGAGGATCCCCGGGAACAAAAACTTATTTCTGA-3′
C6	C6.GAP.AS	5′-TCAGAAATAAGTTTTTGTTCCCGGGGATCCTCTAGAGCAAAATGATAAATACAGACATCGTAAACC-3′
C7	C7.GAP.S	5′-TCTCCAGTCAACTGGTCTTTACCAGTTTATGCTCTAGAGGATCCCCGGGAACAAAAACTTATTTCTGA-3′
C7	C7.GAP.AS	5′-TCAGAAATAAGTTTTTGTTCCCGGGGATCCTCTAGAGCATAAACTGGTAAAGACCAGTTGACTGGAGA-3′
C8	C8.S	5′-AAGGAAATGATTATGAGAGCCTGG-3′
C8	C8.AS	5′-ACCCTGATAAATACAGACATCG-3′

[a] The bases written in lower case indicate introduced restriction sites (ccgcgg for SacII) as well as extensions flanking these sites.

nylidene difluoride membrane (Hybond P, Amersham Biosciences). The membrane was blocked with PBS and 5% milk before incubation with the mouse monoclonal antibody anti-c-*myc* (clone 9E10, Roche) at a 1:400 dilution in PBS and 5% milk for 1.5 h. After a wash in blocking solution, the membrane was incubated with the secondary antibody, goat anti-mouse IgG horseradish peroxidase (Promega), at a 1:2500 dilution in PBS and 5% milk for 1 h. The membrane was washed in a blocking solution of PBS and 0.1% Tween, and finally PBS. Western blot membranes were developed using ECL Plus Western blotting detection reagent (Amersham Biosciences), 0.12 ml/cm^2, and visualized by the LAS-100 image reader (Fuji Film). Western blot analysis of Hog1p phosphorylation of Thr^{174} and Thr^{176} was prepared as described previously (24).

Glycerol Accumulation Measurements—For glycerol accumulation measurements, cells were pregrown in YNB supplemented with 2% glucose to mid-log phase (OD_{600} of 0.5–0.7) and collected by centrifugation at 3000 × *g* for 5 min at room temperature. At $t = 0$, cells were resuspended in medium with 1.5 M sorbitol. 2-ml aliquots were withdrawn at $t = 0.5$, 1, 2, 3, 4, 6, 8, 10, and 24 h for total and intracellular glycerol as well as dry weight determination. The two latter samples were filtered on Whatman GF/C filters. Filters for dry weight determination were dried at 80 °C for 16 h, and filters for intracellular glycerol measurements were soaked in 0.5 M Tris-HCl, pH 7. Glycerol samples were heated at 100 °C for 10 min, and the glycerol concentration in the supernatant after centrifugation (10,000 × *g*, 10 min, room temperature) was determined using a Biomek 2000 laboratory robot (Beckman Coulter). Three transformants from each construct were tested, and the mean value ± S.E. ($n = 3$) was calculated for each time point.

RESULTS

Truncations in the C terminus of Fps1p Confer an Osmosensitive Phenotype—A set of eight truncations in the C-terminal extension of Fps1p was constructed (Fig. 1*B*) and expressed in yeast cells lacking the endogenous *FPS1* gene. Growth assays on plates revealed that all of the transformants grow like the control strains in YNB medium supplemented with 2% glucose. In medium with 1 M sorbitol, *i.e.* high osmolarity, growth was significantly diminished for cells expressing C1 ($Fps1p^{\Delta534–650}$). These transformants expressed Fps1p lacking the major part of the C terminus (Fig. 2*A*). The growth defect for C1 ($Fps1p^{\Delta534–650}$) was somewhat less pronounced than that observed previously for N-terminal truncations, of which the *fps1*-Δ1 ($Fps1p^{\Delta12–231}$) truncation causes the largest effect (Fig. 2*B*). In the latter case, the growth defect was shown to be due to an unregulated channel and the concomitant loss of glycerol from the cell (13). Growth curves revealed that the increased osmosensitivity of C1 ($Fps1p^{\Delta534–650}$) was due to both a longer generation time and a lower yield of cells (Fig. 2*B*). Because survival under these conditions is dependent on proper Fps1p control, growth defects under hyper-osmotic conditions indicated an unregulated Fps1 protein. Hence, it appears that the C terminus is needed for proper channel regulation, analogous to previous observations for the N terminus.

In an attempt to narrow down the region within the C terminus that is important for channel control, we have constructed a set of seven additional, overlapping mutations, C2-C8 (Fig. 1*B*). Plate growth assays show that, under hyper-osmotic conditions, the growth of cells expressing C2 ($Fps1p^{\Delta577–650}$), C4 ($Fps1p^{\Delta546–650}$), C5 ($Fps1p^{\Delta649}$), and C8 ($Fps1p^{\Delta534–547}$) were not significantly different from the growth of cells expressing the wild type gene *FPS1*, whereas the growth of cells expressing C3 ($Fps1p^{\Delta534–578}$), C6 ($Fps1p^{\Delta534}$), and C7 ($Fps1p^{\Delta546}$) were affected to different extents (Fig. 2*A*).

To analyze the functionality of the C-terminal truncations, the expression level and the membrane localization of each mutant were determined. Cellular fractionation demonstrated that most of the truncated proteins were detected in the membrane fraction (Fig. 2*C*). Large truncations in the C terminus resulted in a low level of Fps1p in the membrane. This was especially pronounced for the C6 ($Fps1p^{\Delta534}$) and C7 ($Fps1p^{\Delta546}$) constructs, in which case the amount was very low or below detection in the total membrane fraction. One explanation for this apparently low expression might be that the myc tag is less accessible when located closer to the transmembrane domain. Even the apparently poorly expressed or poorly detectable C6 ($Fps1p^{\Delta534}$) and C7 ($Fps1p^{\Delta546}$) constructs were functional. This is illustrated by the ability to complement the hypo-osmotic shock sensitivity of the *fps1*Δ mutant, suggesting sufficient glycerol efflux to allow survival and growth. In this test, cells transformed with *FPS1* or any of the truncated variants had ~10 times improved growth compared with cells transformed with the vector (Fig. 2*A*).

C-terminal Truncations of Fps1p Confer a Defect in Glycerol Accumulation—To investigate the reason for the hyper-osmosensitivity conferred by the C-terminal truncations, we monitored the accumulation of glycerol under hyper-osmotic conditions. As shown in Fig. 3*A*, cells expressing the wild type *FPS1* gene rapidly accumulated intracellularly produced glycerol under hyper-osmotic conditions. As shown previously, expression of *fps1*-Δ1 ($Fps1p^{\Delta12–231}$) resulted in poor accumulation due to a high channel activity (13). Deletion of the major part of the C terminus (C1, $Fps1p^{\Delta534–650}$) also resulted in an inefficient accumulation of intracellular glycerol, but the effect was somewhat less pronounced, consistent with growth data (Fig. 2*B*). Retaining 43 aa (C2, $Fps1p^{\Delta577–650}$) close to the last TMD resulted in an accumulation profile comparable with wild type, although deletion of just these residues (C3, $Fps1p^{\Delta534–578}$) resulted in an intermediate glycerol accumulation capacity. Keeping a stretch of 12 aa close to the last TMD (C4,

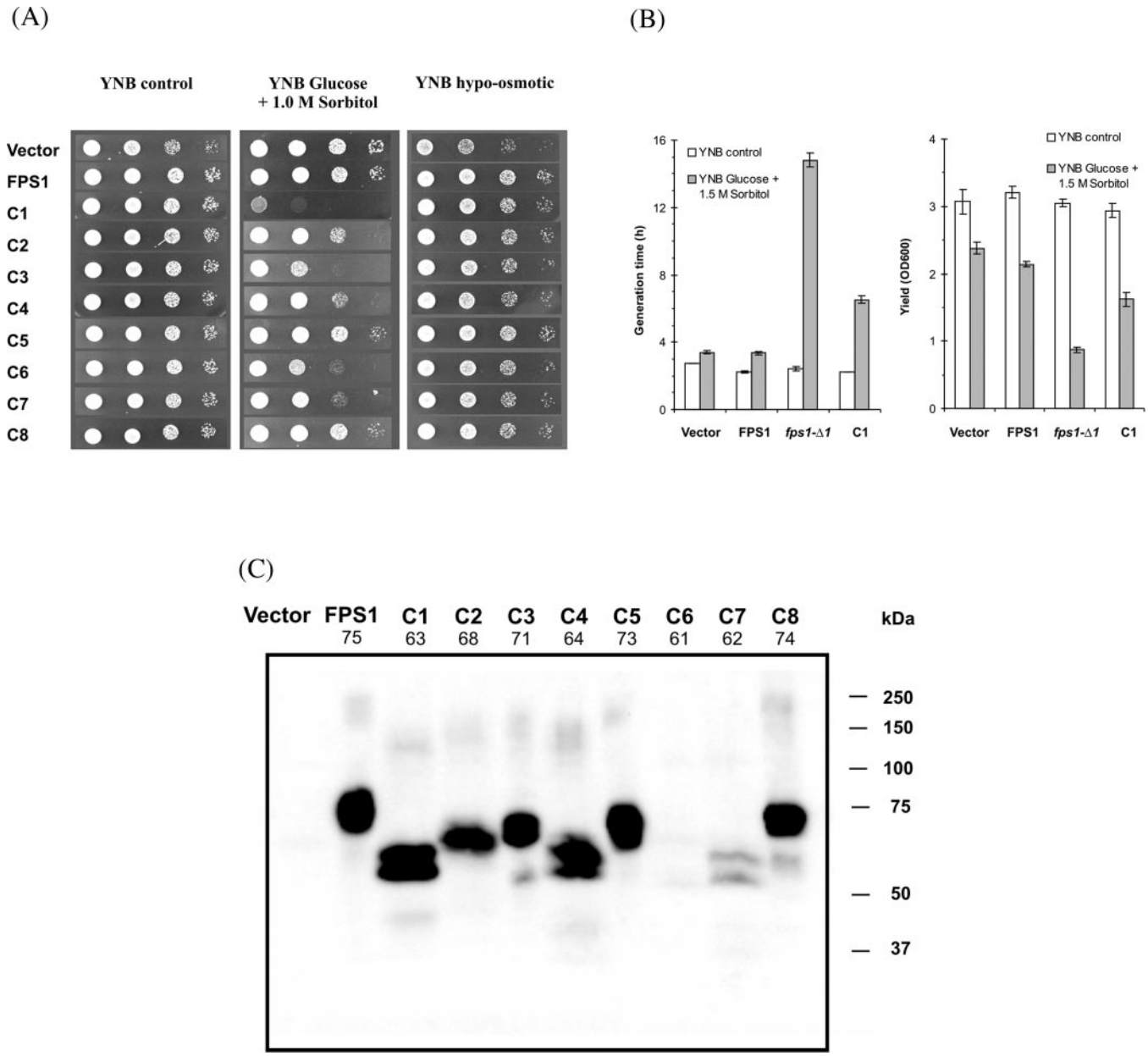

FIG. 2. **Growth characteristics following an osmotic shock and membrane localization of truncated proteins.** *A*, plate growth assay under hyper-osmotic conditions and following a hypo-osmotic shock. For growth assays, the *fps1*Δ mutant was transformed with an empty plasmid, the wild type gene, or the eight C-terminal truncation constructs. Serial dilutions of cells were spotted on normal medium as a control. For a hypo-osmotic shock, cells were spotted on medium with 1.0 M sorbitol as a control and on normal medium for a hypo-osmotic shock. Transformants were spotted in triplicates, and typical series are shown. *B*, growth in normal and high osmolarity medium. The generation time was calculated from the logarithmic phase of the growth. Three transformants from each construct were grown. The mean value ± S.D. ($n = 3$) is shown for each transformant. *C*, Western blot analysis of total membrane extracted from yeast cells expressing Fps1p and variants. A representative blot is shown, and the estimated molecular mass for each protein is indicated below the name of the construct. 10 μg of total protein was loaded for *FPS1*, C3, C5 and C8, 100 μg was loaded for vector, C1, C2, C4, and 400 μg was loaded for C6 and C7.

Fps1p$^{\Delta 546-650}$ and C7, Fps1p$^{\Delta 546-}$) resulted in an initial accumulation capacity comparable with that of wild-type Fps1p, whereas deletion of these 12 aa (C6, Fps1p$^{\Delta 534}$) gave rise to as poor an initial accumulation as for *fps1*-Δ1 (Fps1p$^{\Delta 12-231}$) (Fig. 3*A*).

The profiles of intracellular accumulation (Fig. 3*A*) and the total glycerol production (Fig. 3*B*) were in good agreement with the growth data (Fig. 2*A*). This means that a poor growth rate at high osmolarity was associated with reduced glycerol accumulation and higher levels of total glycerol produced. With glycerol overproduction, the cell attempts to compensate for the poor ability to retain glycerol in the cell. We noted that C6 (Fps1p$^{\Delta 534-}$), the largest truncation, caused similar poor glycerol accumulation and high total glycerol production as C1 (Fps1p$^{\Delta 534-650}$). Taken together, the glycerol accumulation and production profiles confirmed that the truncations C1 (Fps1p$^{\Delta 534-650}$), C3 (Fps1p$^{\Delta 534-578}$), C6 (Fps1p$^{\Delta 534-}$), and C7 (Fps1p$^{\Delta 546-}$) render Fps1p unregulated to different extents.

C-terminal Truncations of Fps1p Affect the Profile of Osmostress Signaling—To examine whether C-terminal truncations gave rise to an unregulated Fps1p, we monitored the profile of high osmolarity glycerol (HOG) pathway activation in cells expressing C1 (Fps1p$^{\Delta 534-650}$) and C2 (Fps1p$^{\Delta 577-650}$). High osmolarity mediates phosphorylation of the mitogen-activated protein kinase Hog1p, which, in turn, stimulates expression of genes encoding enzymes in glycerol biosynthesis (11). The defect in glycerol accumulation conferred by an unregulated, N-terminally truncated Fps1p has previously been shown to be

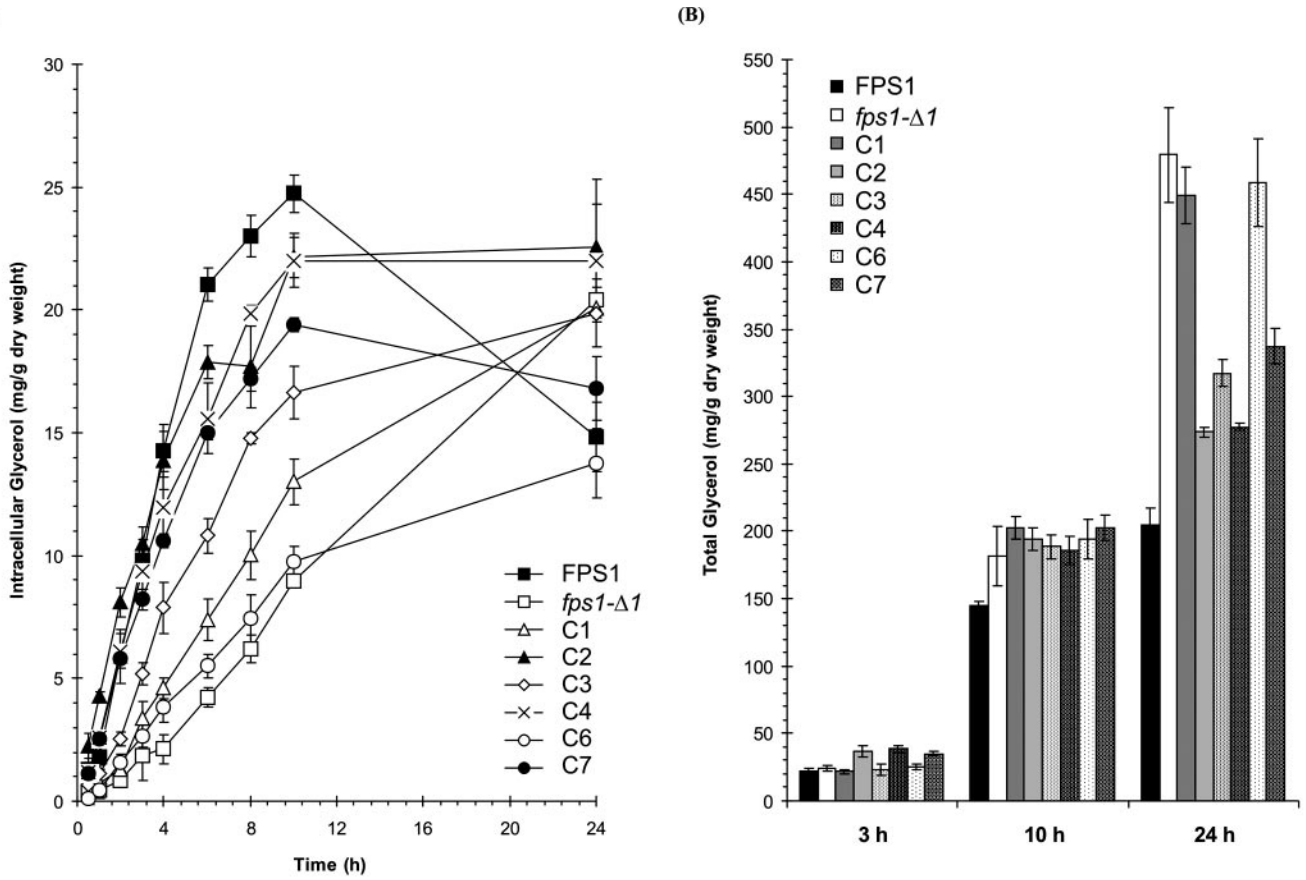

FIG. 3. **Glycerol accumulation following hyper-osmotic shock and support for unrestricted channels.** For glycerol accumulation measurements, cells were shifted from YNB medium supplemented with 2% glucose to the same medium with 1.5 M sorbitol at $t = 0$. Glycerol accumulation was monitored over a period of 24 h. *A*, intracellular glycerol, the mean value ± S.E. ($n = 3$) at $t = 0.5, 1, 2, 3, 4, 6, 8, 10$, and 24 h, for cells of the *fps1*Δ mutant transformed with the wild type gene and truncated forms. *B*, total glycerol, the mean value ± S.E. ($n = 3$), at $t = 3$, 10, 24 h.

associated with extended phosphorylation of the mitogen-activated protein kinase Hog1p, which is a good indicator of the status in the cell.[2] Such sustained phosphorylation was also observed in cells expressing C-terminally truncated Fps1p (Fig. 4). The period of Hog1p phosphorylation was prolonged in cells expressing the C1 ($Fps1p^{\Delta 534-650}$) truncation, whereas the phosphorylation profile of cells expressing C2 ($Fps1p^{\Delta 577-650}$) was similar to that of cells expressing the wild type *FPS1* gene. These results are consistent with the growth and glycerol accumulation data that demonstrated a strong effect of the long C1 ($Fps1p^{\Delta 534-650}$) truncation. Hence, the osmostress signaling profile confirmed that truncations of the C terminus render Fps1p unregulated.

C-terminal Truncation of Fps1p Confers Xylitol Uptake—A sensitive assay for an unregulated Fps1p is its ability to confer growth to a *gpd1*Δ *gpd2*Δ mutant on medium containing a high concentration of xylitol.[2] In contrast to sorbtiol, xylitol can be taken up via an unregulated Fps1p channel and act as an internal osmolyte. The *gpd1*Δ *gpd2*Δ double mutant is unable to produce glycerol and is, for this reason, very sensitive to high osmolarity, for instance with 1 M xylitol. Expression of an unregulated Fps1p allows inflow of xylitol to the cell, relieving the osmotic stress and allowing growth. We tested the C-terminal truncations in this system and found that truncations C1 ($Fps1p^{\Delta 534-650}$), C3 ($Fps1p^{\Delta 534-578}$), C6 ($Fps1p^{\Delta 534-}$), and C7 ($Fps1p^{\Delta 546-}$) conferred growth of the *gpd1*Δ *gpd2*Δ mutant on xylitol (Fig. 5*A*). This result is consistent with the notion that C-terminally truncated Fps1p is unregulated.

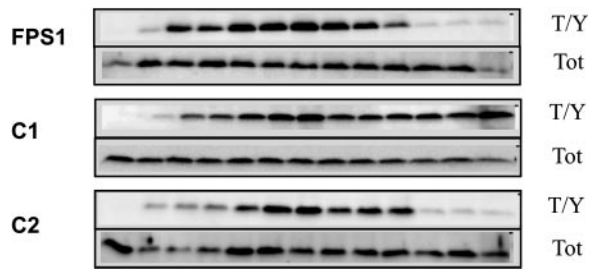

FIG. 4. **Determination of Hog1p phosphorylation was carried out by Western blot analysis using antibodies against dual-phosphorylated Hog1p (*T/Y*) and total Hog1p (*Tot*) in the fps1Δ mutant transformed with the wild type gene, C1, and C2.** Cells were shifted from YNB medium supplemented with 2% glucose to the same medium containing 1.5 M sorbitol. Samples were taken at the indicated time points (0, 1, 5, 10, 20, 30, 40, 50, 60, 90, 120, 180, and 300 min).

DISCUSSION

In this work we report a novel function for the C-terminal domain of Fps1p, an atypical member of the MIP family. Cells expressing a C-terminally truncated Fps1p show sensitivity to hyper-osmotic conditions, which indicates a role for the C terminus in channel regulation. Expression of this channel results in delayed intracellular glycerol accumulation under hyper-osmotic conditions, which is compensated by glycerol overproduction. The fact that truncations of the Fps1p C terminus cause an unregulated channel is further supported by prolonged phosphorylation of Hog1p as well as improved growth of

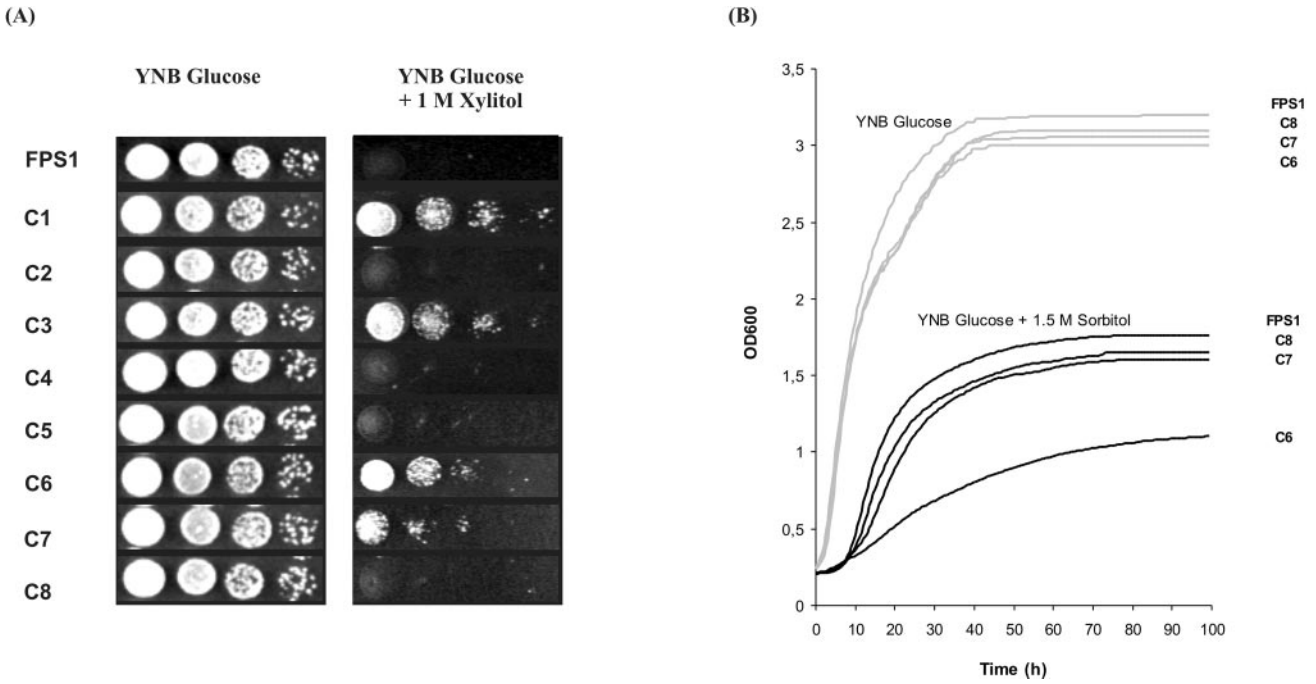

FIG. 5. **Characterization of truncations introducing sensitivity to hyper-osmotic conditions.** *A*, growth advantage for an unregulated Fps1p channel on xylitol showed for *FPS1*, C1-C8 expressed in the *gpd1*Δ *gpd2*Δ strain. *B*, growth in normal and high osmolarity medium. The average growth curves for three transformants from each construct are shown.

the *gpd1*Δ *gpd2*Δ mutant on xylitol as a result of expressing Fps1p lacking its C terminus.

The difference in growth sensitivity to high omsolarity, glycerol accumulation, total glycerol production, and phosphorylation profiles of Hog1p, as well as growth of the *gpd1*Δ *gpd2*Δ mutant on xylitol caused by truncations C1 ($Fps1p^{\Delta534-650}$) and C2 ($Fps1p^{\Delta577-650}$), points to an important regulatory domain close to the last TMD. Deletion of just this domain of 43 aa (C3, $Fps1p^{\Delta534-578}$) gives rise to a partially regulated channel, as illustrated by an intermediate capacity to accumulate intracellular glycerol (Fig. 3*A*) as well as improved growth on xylitol in the *gpd1*Δ *gpd2*Δ background (Fig. 5*A*). This activity resulted in an intermediate sensitivity to hyper-osmotic conditions for transformants expressing this construct (Fig. 2*A*). This might indicate that other parts of the C-terminal extension partly compensate for this critical function when moved closer to TMD6. The important domain could be shortened from 43 to 12 amino acids without affecting function; C4 ($Fps1p^{\Delta546-650}$) transformants were also not significantly sensitive to hyper-osmotic conditions (Fig. 2*A*). The C4 ($Fps1p^{\Delta546-650}$)-encoded protein seemed to form functional glycerol efflux channels (Fig. 2*A*) with glycerol accumulation profiles similar to those for wild type Fps1p (Fig. 3). An additional truncation (C5, $Fps1p^{\Delta649-}$) was made to test whether the most distal 20 aa, which were retained in all constructs, could form a regulatory domain. As seen in Figs. 2*A* and 5*A*, C5 ($Fps1p^{\Delta649-}$) did not confer any sensitivity to hyper-osmotic conditions, seemed to form functional glycerol efflux channels when expressed in the *fps1*Δ strain, and did not confer improved growth on xylitol when expressed in the *gpd1*Δ *gpd2*Δ background. Because the last 20 aa were not crucial for proper regulation, it suggested that the difference between a regulated and an unregulated Fps1p could be explained by the properties of the 12 residues next to the last TMD (Fig. 1*B*). In agreement with this hypothesis, C6 ($Fps1p^{\Delta534-}$) caused growth sensitivity to severe hyper-osmotic conditions (1.5 M sorbitol), whereas C7 ($Fps1p^{\Delta546-}$) did not (Fig. 5*B*). When stressed with 1.0 M sorbitol, the effect as well as the difference between the two constructs was less pronounced, which might be explained by a very low level of production (Fig. 2). Both C6 ($Fps1p^{\Delta534-}$)- and C7 ($Fps1p^{\Delta546-}$)-expressing strains seemed to export glycerol upon a hypo-osmotic shock indicative of a functional channel (Fig. 2). A clear difference was observed for C6 ($Fps1p^{\Delta534-}$) and C7 ($Fps1p^{\Delta546-}$) in the intracellular as well as the total glycerol accumulation (Fig. 3, *A* and *B*), verifying the apparently crucial role of these 12 residues (^{535}HESPVNWSLPVY^{546}) in channel regulation. Furthermore, expression of C6 ($Fps1p^{\Delta534-}$) in the *gpd1*Δ *gpd2*Δ strain resulted in improved growth in the presence of xylitol, indicating that this protein is an unrestricted channel (Fig. 5*A*). Some xylitol uptake was also seen for C7 ($Fps1p^{\Delta546-}$), supporting the notion that this protein does not have full regulatory capacity despite the fact that it is equipped with the important domain for regulation. However, simple deletion of the latter domain of 12 aa (C8, $Fps1p^{\Delta534-547}$) did not affect channel regulation (Figs. 2*A* and 5*A*); but it is not unreasonable that, for C8 ($Fps1p^{\Delta534-547}$) as well as for C3 ($Fps1p^{\Delta534-578}$), the bulk of the remaining C-terminal sequence compensates for the truncation.

In conclusion, our results indicate that a short C-terminal sequence of 12 residues close to the last transmembrane domain has an important role in channel regulation. This domain shows similarities to the N-terminal restriction domain and has a potential to dip into the membrane. The C-terminal 12 residues long domain is ^{535}HESPVNWSLPVY^{546} (Fig. 1*A*). The reverse N*XX*L motif, L*XX*N, was previously found in the corresponding N-terminal domain, ^{225}LYQNPQTPTVLP^{236} (Fig. 6), in which the leucine and the asparagine were shown to be important for proper channel regulation (17). Both regulatory N- and the C-terminal domains are proline-rich, suggesting that the hydrophilic domains have a structural influence on the protein. The fact that the N terminus restricts transport through Fps1p and has an amphiphilic character suggests that it dips into the membrane (17). The same may be valid for the C-terminal sequence, where the leucine and valine residues may have a major influence on membrane insertion.

We have previously postulated that either of the glutamine residues (Gln^{227} or Gln^{230}) of the N-terminal regulatory domain of Fps1p may interact with His^{350} of loop B, close to the

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-Q-P-NQYLTKPKVMIPV
HESPVNWSL--P-VY
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FIG. 6. **Sequence similarity between N-terminal (sequence reversed) and C-terminal regulatory domains found by truncation analysis.**

extramembrane face (17). For the C-terminal regulatory domain, an equivalent possibility is Glu^{536} interacting with Arg^{363} of loop B, again at a shallow depth in the pore. This would allow Asn^{540} to come into close proximity with the NP motif of loop B and, possibly, also the NP motif of the N-terminal regulatory domain. It is possible that the N-terminal and C-terminal regulatory domains permanently lie in different parts of the pore and associate with different parts of loop B or, alternatively, that they may flip in and out of the membrane independently according to conditions. Thus, conformational changes may be responsible for fine regulation of pore activity. These hypotheses are currently examined through the construction of a series of point mutations and a genetic screen.

The activity of a number of eukaryotic MIPs has been shown to be regulated by diverse mechanisms such as pH (25–27), phosphorylation to induce protein trafficking, or direct channel gating (28, 29) and expression (30–33). In conclusion, there is considerable diversity within the MIP family, and a common theme for a regulatory mechanism is lacking. The expression of Fps1p is not regulated by a hyper-osmotic shock, although it may be affected by long-term adaptation to osmotic changes (13). A unique regulatory mechanism has been presented for Fps1p activated by shifts in external osmolarity in which the long hydrophilic extensions in both termini have been suggested to be crucial for control of the channel function. The regulatory mechanism is not yet understood, even though several possible explanations have been excluded (13, 34). It is believed that the mechanism of regulation is intrinsic to the protein and involves integral structural rearrangement. Despite the high identity found between Fps1p and other MIPs, especially GlpF, there is no known candidate within the family that has the same characteristics regarding the response to osmotic changes.

The N- and the C-terminal regions found to be of major importance in controlling the Fps1p function are relatively short and, in both cases, they are located close to TMD1 and TMD6, respectively. When these domains are deleted, the Fps1p channel cannot restrict transport upon high osmolarity. The Fps1p N terminus has been demonstrated to restrict glycerol transport, both under normal and hyper-osmotic conditions. A similar function for the C terminus is indeed suggested by the present results. Point mutations within the C-terminal domain identified in this study are being constructed to evaluate the mechanism of regulation, and a pure Fps1p (23) reconstituted into proteoliposomes is currently being assayed to gain a deeper insight into the mechanism of regulation of this atypical MIP.

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A Regulatory Domain in the C-terminal Extension of the Yeast Glycerol Channel Fps1p

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