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A Short Regulatory Domain Restricts Glycerol Transport through Yeast Fps1p*

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The controlled export of solutes is crucial for cellular adaptation to hypotonic conditions. In the yeast *Saccharomyces cerevisiae* glycerol export is mediated by Fps1p, a member of the major intrinsic protein (MIP) family of channel proteins. Here we describe a short regulatory domain that restricts glycerol transport through Fps1p. This domain is required for retention of cellular glycerol under hypertonic stress and hence acquisition of osmotolerance. It is located in the N-terminal cytoplasmic extension close to the first transmembrane domain. Several residues within that domain and its precise position are critical for channel control while the proximal residues 13–215 of the N-terminal extension are not required. The sequence of the regulatory domain and its position are perfectly conserved in orthologs from other yeast species. The regulatory domain has an amphiphilic character, and structural predictions indicate that it could fold back into the membrane bilayer. Remarkably, this domain has structural similarity to the channel forming loops B and E of Fps1p and other glycerol facilitators. Intragenic second-site suppressor mutations of the sensitivity to high osmolarity conferred by truncation of the regulatory domain caused diminished glycerol transport, confirming that elevated channel activity is the cause of the osmosensitive phenotype.

Accumulation of osmolytes is a ubiquitous strategy of cellular osmoadaptation (1). Cells produce or actively take up osmolytes in order to increase their solute content and thereby maintain turgor and volume under hypertonic conditions (high extracellular osmolarity). Upon shift to hypotonic conditions, *i.e.* when the extracellular osmolarity drops, cells export solutes to prevent excessive swelling or bursting in a process termed regulated volume decrease (2). While proteins that mediate solute export from mammalian cells have not been identified yet, such channels have been described in bacteria and yeast (3–6). The MscL channel from *Escherichia coli* has been particularly well studied, and the structural basis for gating by osmotic changes has been discussed in detail (5, 7–11).

Proliferating yeast (*Saccharomyces cerevisiae*) cells employ glycerol as osmolyte, which they produce in two steps from the glycolytic intermediate dihydroxyacetonephosphate (12). We have previously demonstrated that Fps1p mediates export of glycerol across the yeast plasma membrane (13–16). Mutants lacking Fps1p are unable to rapidly export glycerol upon hypoosmotic shock and only a fraction of such cells survive under these conditions (14, 15). We have also provided evidence that transmembrane glycerol flux is regulated by osmotic changes. Glycerol flux is diminished within seconds after a shift to high osmolarity and it increases again at an apparently similar time scale when cells are shifted to low osmolarity (15). The N-terminal extension of Fps1p appears to be required for controlling glycerol transport as its deletion renders the channel hyperactive. Yeast cells expressing such a hyperactive channel are sensitive to high osmolarity because they need much longer to build up high intracellular glycerol levels due to a higher level of glycerol leakage (15).

Fps1p belongs to the MIP[1] (major intrinsic protein) family of channel proteins. Members of this ancient family have been identified in organisms ranging from Archea to human (17–20). MIP channels comprise water channels (aquaporins) and glycerol facilitators (aquaglyceroporins) (17). Water channels are highly specific to water (17, 21, 22), although transport of ions has also been reported (23, 24). Glycerol facilitators commonly transport small polyols and a range of other uncharged molecules (25), and apparently even metalloid ions (26, 27). The three-dimensional structure of human aquaporin AQP1 and of the *E. coli* glycerol facilitator GlpF have been determined (21, 22, 25, 28). MIP channels consist of six transmembrane domains (TMDs) comprised of an internal repeat of three TMDs. Loops B and E form two half TMDs that interact within the membrane and are part of the selective pore. These two loops contain the canonical NPA (asparagine-proline-alanine) motifs

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[1] The abbreviations used are: MIP, major intrinsic protein; MES, 4-morpholineethanesulfonic acid; TMD, transmembrane domain.

TABLE I
Primers used for mutagenesis

Name of primer	Sequence 5′–3′
P222A	CCCATTATGGTGAAGGCAAAGACATTATACCAG
T224A	ATGGTGAAGCCAAAGGCATTATACCAGAACCCT
L225A	TGAAGCCAAAGACAGCATACCAGAACCCTC
Y226F	GTGAAGCCAAAGACATTATTCCAGAACCCTCAAACACCT
Q227A	CAAAGACATTATACGCGAACCCTCAAACAC
N228A	GACATTATACCAGGCCCCTCAAACACCTAC
P229A	CCAAAGACATTATACCAGAACGCTCAAACACCTACAGTC
Q230A	TACCAGAACCCTGCAACACCTACAGTCT
T231A	TACCAGAACCCTCAAGCACCTACAGTCTTGCCC
P232A	CAGAACCCTCAAACAGCTACAGTCTTGCCCTCC
T233A	CAGAACCCTCAAACACCTGCAGTCTTGCCCTCCACA
P236A	CAAACACCTACAGTCTTGGCCTCCACATACCATCCA
S237A	ACACCTACAGTCTTGCCCGCCACATACCATCCA
T238A	CCTACAGTCTTGCCCTCCGCATACCATCCAATT
M5f.	CCTACTCCCACGTATGTTCCA
M6r.	CAGGGGAAAACCTCTATACACC

that are part of the MIP channel signature sequence, which is conserved in almost all of the presently known 300 MIP channels (17, 29, 30). Fps1p is an unusual MIP channel. The NPA motifs in loops B and E are replaced by NLA and NPS, respectively. This observation together with mutational analyses suggests that the channel architecture of Fps1p differs from that of other glycerol facilitators (31). In addition, Fps1p has unusually long N- and C-terminal extensions of 255 and 139 amino acids, respectively. These extensions have no obvious similarity to other proteins in the databases.

In this study we identified a regulatory domain within the N-terminal extension. It is located close to the first TMD and restricts transmembrane glycerol flux both under high osmolarity and under normal growth conditions. The regulatory domain shows structural similarity to the channel forming loops B and E suggesting that it might itself dip into the membrane. We discuss implications for the mechanisms by which this domain could be involved in controlling channel function.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—The *S. cerevisiae* strains used in this study were wild-type W303-1A (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*) (32) and an isogenic *fps1*Δ::*HIS3* (YMT2). Yeast cells were routinely grown on a rotary shaker at 30 °C in YNB (yeast nitrogen base) medium (33) containing 2% glucose as a carbon source.

Plate growth assays were performed by pregrowing cells either in medium without salt (for hyperosmotic shock) or in medium supplemented with 1 M sorbitol (for hypo-osmotic shock). Cells were suspended in the same medium to an OD_{600} of 1.0. Five microliters of each dilution of 10-fold serial dilutions were spotted onto agar plates supplemented with 2% glucose and 0.8 M NaCl (hyperosmotic shock) or lacking osmoticum (hypo-osmotic shock). Growth was monitored after 2–3 days at 30 °C.

Construction of FPS1 Mutants—YEp*myc-FPS1* is a 2μ *LEU2* plasmid expressing a c-*myc* epitope-tagged Fps1p (15). The *FPS1* alleles containing larger truncations (constructs *fps1-Δ6* to *fps1-Δ11*), pairwise amino acid replacements (constructs *fps1-12* to *fps1-24*) or insertions/deletions (constructs *fps1-25* to *fps1-28*) were constructed by completely amplifying YEp*myc-FPS1* except for the region to be deleted or altered (15). The primers used contained either a *Sac*II site (constructs *fps1-Δ6* to *fps1-Δ11*) or a *Spe*I site (constructs *fps1-12* to *fps1-28*) and three additional nucleotides at their 5′-end. Ligation results in the insertion of a proline and an arginine residue (*Sac*II) or a threonine and a serine residue (*Spe*I) at the site of the deletion. All constructs were confirmed by sequencing.

FPS1 alleles containing alanine or phenylalanine point mutations were constructed using the megaprimer polymerase method (34) with YEp*myc-FPS1* as template and the mutagenesis and flanking (M5 and M6) primers listed in Table I. The resultant PCR products were cotransformed into *S. cerevisiae* with *Kpn*I- and *Csp*I-digested YEp*myc-FPS1*. The resulting gap-repaired plasmids (35) were propagated in *E. coli* TOP 10F′ and confirmed by sequencing. All other molecular biological manipulations were performed using standard techniques (36).

Glycerol Levels and Glycerol Transmembrane Flux—Intracellular glycerol levels were determined essentially as described previously (15). To determine the proportion of produced glycerol that is retained, cells were grown in liquid YNB medium to an OD_{600} of 0.5–1.0, sedimented, and resuspended in medium containing 0.8 M NaCl, and samples were collected by filtration.

To determine glycerol influx following its concentration gradient, cells were grown in liquid YNB medium to an OD_{600} of ~2.0. Cells were harvested, washed, and suspended in ice-cold MES buffer (10 mM MES, pH 6.0) to a density of 40–60 mg of cells ml^{-1}. Glycerol influx in the presence or absence of hyperosmotic stress was measured by adding glycerol to a final concentration of 100 mM "cold" glycerol plus 40 μM [^{14}C]glycerol (160mCi/mmol; Amersham Biosciences) in a total volume of 250 μl (15). Aliquots of 50 μl were collected by filtration and washed twice, and the radioactivity retained on the filters was determined. Filters with cells were dried at 80 °C overnight for dry weight determination. Transport experiments were performed in triplicate.

Membrane Preparation and Western Analysis—Yeast membranes were prepared as previously described (15). 10 μg of total protein were separated by SDS-PAGE and blotted onto nitrocellulose filters. The filters were probed with mouse monoclonal anti-c-*myc* (9E10, Santa Cruz Biotechnology) as primary antibody and alkaline phosphatase-conjugated anti-mouse IgG as secondary antibody. For detection, the membrane filters were incubated with 50 mg of 5-bromo-4-chloro-3-indolyl phosphate and 75 mg of nitroblue tetrazolium salts per ml. Protein was quantified using the method of Bradford (37) with bovine serum albumin as standard.

Structural Predictions—Sequence alignments were carried out using ClustalW (38). Mean values for hydrophobicity were obtained as described (39, 40). Propensity for α and β conformation was predicted by a sliding window calculation of the cumulative index for three successive residues, using previously reported scales (41). The models of the putative membrane dipping regions were generated by extraction of the Cα atom coordinates of loop B of *E. coli* GlpF from the Brookhaven Protein Data bank (PDB) file, 1FX8, using appropriate commands in RASMOL (42), followed by replacement with corresponding amino acid residues of the aligned Fps1p regulatory domain and construction of the loop using the MaxSprout algorithm (43). Predicted secondary structure was corroborated by analysis of the modeled structures using TMAlpha (44).

RESULTS

Deletion Analysis of the N-terminal Extension Reveals a Domain Necessary for Control of Fps1p—The predicted Fps1 protein consists of a hydrophilic N-terminal extension (amino acids 1–255), the core of six transmembrane domains (TMDs 1–6) with their connecting loops A–E (amino acids 256–530), and a hydrophilic C-terminal extension (amino acids 531–669) (13, 31). Previously we have shown that deletion of the N-terminal extension between positions 13 and 230 renders Fps1p hyperactive (15). Deletion of residues 76–230 and 151–230 also increased transport through Fps1p while truncation upstream of position 145 did not cause any obvious effect. These data indicated that the segment important for control of Fps1p is located between positions 150 and 231 (15).

To define this regulatory domain in more detail we generated a further six deletions (Fig. 1*A*). Those deletions were constructed in an *FPS1* gene cloned under control of its own promoter into a multicopy plasmid and then expressed in an *fps1*Δ mutant. All constructs carried a C-terminal c-*myc* tag. By Western blot analysis of membrane preparations we confirmed that all constructs were expressed and that the gene products were located in fractions containing the plasma membrane (Fig. 1*C*). The amount of protein located in the plasma membrane seemed to differ between different alleles. Since we did not observe any correlation between the apparent amounts of membrane-localized Fps1p and functionality we assumed that the different protein levels did not affect the interpretation of the experiments. Similar observations were reported previously (31). For unknown reasons in some samples two bands

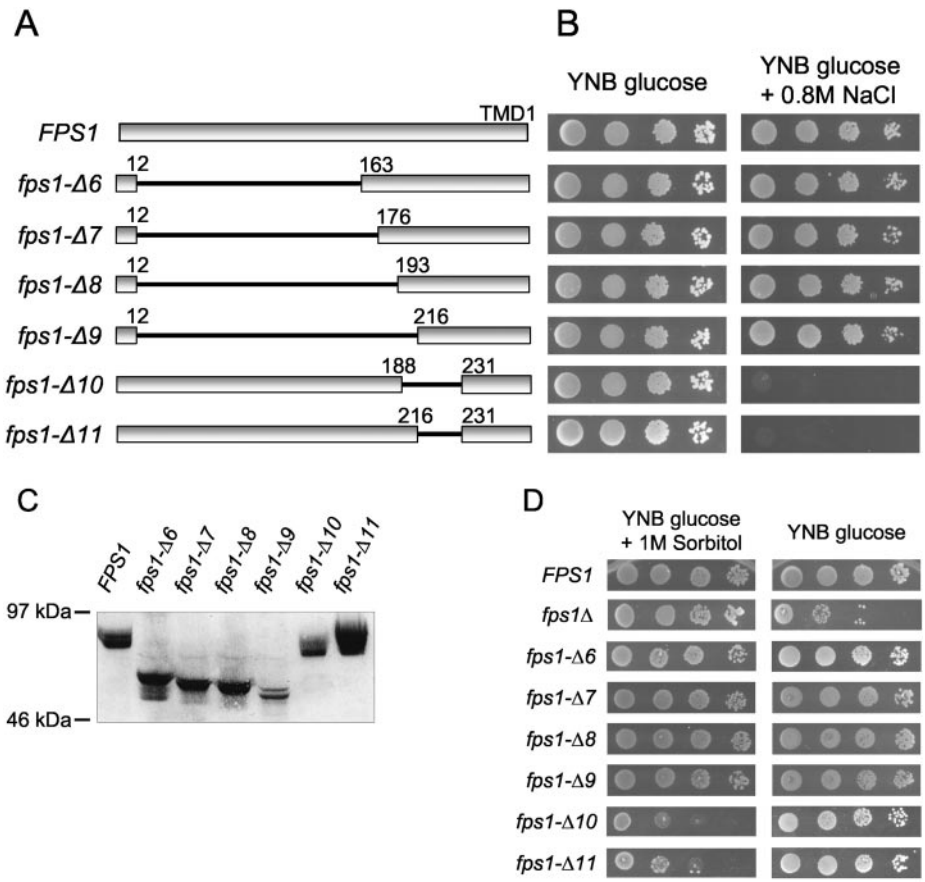

FIG. 1. **Deletion analysis of the N-terminal extension of Fps1p.** *A*, sketch of the N-terminal extension indicating the endpoints of deletions. *TMD1*, first transmembrane domain. *B*, growth phenotypes of the *fps1*Δ mutant transformed with plasmids carrying the different deletion constructs. Serial 1:10 dilutions starting with a cell suspension of $OD_{600\ nm}$ = 1 were spotted on synthetic YNB medium with and without 0.8 M NaCl. Failure to grow on salt medium indicates expression of an unregulated channel. *C*, Western blot analysis of cell membrane preparations probed with an anti-c-*myc* antibody raised against the C-terminal c-*myc* tag attached to each deletion construct. All constructs are expressed and membrane localized, albeit at different levels. *D*, growth phenotype upon shift from high (1 M sorbitol) to low (no sorbitol) osmolarity of transformants expressing the different deletion constructs. Poor growth after the hypo-osmotic shift or a lower proportion of surviving cells indicates a non-functional Fps1p, as in the *fps1*Δ control strain.

appeared. Neither evidence for phosphorylation (15) nor glycosylation[2] of the protein, which could possibly explain the nature of the two bands, has been obtained so far.

We then tested the functionality of the Fps1p constructs. The *fps1*Δ mutant is sensitive to hypo-osmotic shock because it is unable to rapidly release accumulated glycerol (15). In order to test if the constructs could complement the hypo-osmosensitivity of the *fps1*Δ mutant cells were pregrown in medium containing 1 M sorbitol and then plated in serial dilutions on medium lacking sorbitol (Fig. 1*D*). It appeared that all constructs could complement the hypo-osmosensitivity of the *fps1*Δ mutant and hence encoded functional glycerol export channels located in the plasma membrane.

To test if channel function was controlled normally we tested transformants for hyperosmosensitivity on plates with 0.8 M NaCl. Cells expressing *FPS1* alleles 6–9 grew on high osmolarity plates like transformants carrying wild type *FPS1* (Fig. 1*B*; see also *left panel* in Fig. 1*D*). This indicated efficient retention of glycerol produced by the cell and successful adaptation to high osmolarity and hence normal restriction of Fps1p-mediated glycerol export. The largest such truncation, *FPS1*-Δ*9*, lacks amino acids 13–215, which hence seemed to be dispensable for channel control. However, cells expressing constructs 10 and 11 grew only poorly on high osmolarity plates (Fig. 1, *B* and *D*), very much like we observed previously for cells expressing Fps1p lacking amino acids 13–230 (15). The poor growth on high osmolarity plates was associated with a pronounced delay in building up high intracellular glycerol levels (data not shown). This is in line with previous observations with other N-terminal truncations (15) and indicated an inability to restrict glycerol transport under hyperosmotic stress. Alleles 10 and 11 lack a segment immediately upstream of position 230 and the smallest truncation, *FPS1*-Δ*11*, lacks amino acids 217–230 but retains all 216 amino acids upstream. Hence, amino acids relevant for channel control are contained within the region covered by residues 217–230. All sequences upstream of this position seem to be dispensable for this control, and their function remains unknown for the moment.

Mutagenesis Defines Amino Acids Required for Restricting Glycerol Transport—To further analyze this regulatory domain we scanned the sequence between 217 and 244 (except for residues Ser-237 and Thr-238) by pairwise replacement of amino acids with threonine and serine (Fig. 2). We chose this combination of amino acids because they are fairly neutral in their effect on secondary structure while we expected their insertion to affect the amphiphilic character of the region. All mutated genes were again expressed from the endogenous *FPS1* promoter on a multicopy plasmid. The mutated proteins were detected in the plasma membrane fraction, they were all functional glycerol exporters as judged from their ability to complement the hypo-osmosensitivity of the *fps1*Δ mutant, and they grew like wild type on control plates lacking osmoticum (data not shown).

Several of the mutations conferred strong osmosensitivity (Fig. 2); some did not have any effect at all while others resulted in intermediate sensitivity. Again, we interpret hyperosmosensitivity as an inability to restrict channel function under high osmolarity stress. Mutation of amino acids Pro-217, Ile-218, Met-219, Val-220 caused moderate osmosensitivity while mutation of the subsequent two amino acids caused strong osmosensitivity, indicative of a hyperactive channel. While exchange of amino acids 223 and 224 again did not seem to affect channel control, all mutations in the region 225–232 caused strong osmosensitivity, identifying this area as being of crucial importance. Note that 231 and 232 are threonine-pro-

[2] K. Hedfalk, Roslyn M. Bill, J. Rydström, and S. Hohmann, unpublished data.

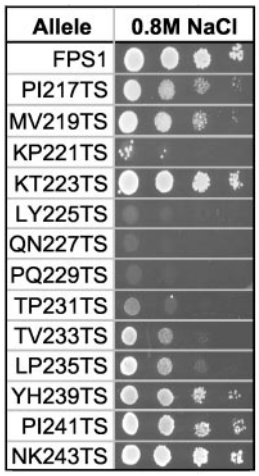

FIG. 2. **Mutagenesis of the regulatory domain by pairwise replacements.** Two adjacent amino acid residues were replaced simultaneously by TS. Poor growth on 0.8 M NaCl indicates expression of a hyperactive channel.

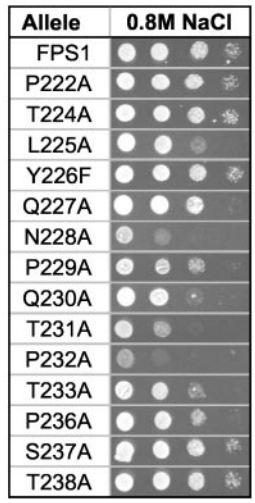

FIG. 3. **Alanine-scanning mutagenesis of the regulatory domain.** Different residues within the regulatory domain were replaced by alanine (or phenylalanine in case of Tyr-226) and expressed in an *fps1*Δ mutant. Failure to grow like wild type on medium with 0.8 M NaCl indicates a hyperactive channel.

line and hence mutagenesis only changed Pro-232. Exchanges at positions 233–236 resulted in partial osmosensitivity suggesting that these residues also contribute to the function of the regulatory domain. Residues 233 and 234 are threonine-valine and hence mutagenesis only affected Val-234.

Based on these observations we decided to focus further mutagenesis on the region between residues 222 and 238. We exchanged individual amino acids to alanine (with the exception of Tyr-226, which was replaced by phenylalanine) and expressed and analyzed the mutant alleles in the same way as described above. All mutated proteins were detected in the plasma membrane fraction, complemented the hypo-osmosensitivity of the *fps1*Δ mutant and grew like wild type on control plates without osmoticum (data not shown).

We noted that the region between 217 and 244 contains a total of six proline residues, which could be of structural importance. Hence we first focused on the prolines in positions 222, 229, 232, and 236. Replacement of Pro-232 caused strong osmosensitivity, confirming the observation from the TS-scanning mutagenesis. However, replacement of Pro-222 had no effect. Since TS replacement of residues Lys-221 and Pro-222 caused strong osmosensitivity it appears that either Lys-221 is critical for channel control and/or that position Pro-222 tolerates alanine but not serine. Replacement of Pro-229 and Pro-236 caused moderate osmosensitivity, suggesting that the exchange with alanine affected channel control only to a certain extent.

We further noted that the region under study contained phosphorylatable amino acids. Previous attempts to link different signaling pathways and protein kinases to channel control had not provided any evidence for a role of phosphorylation of Fps1p in channel regulation (15) but could not exclude such a possibility. We replaced all four threonine residues and the serine residue by alanine and the tyrosine residue by phenylalanine. Only replacement of Thr-231 and Thr-233 caused intermediate or moderate osmosensitivity. These two residues immediately flank the critical Pro-232. Finally, replacement of residues Asn-228, Gln-230, and Leu-225 resulted in different degrees of osmosensitivity. Taken together, it appears that residues Leu-225, Asn-228, Gln-230, Thr-231, and Pro-232 are of particular importance for channel control.

We then chose a subset of the mutants representing different types of mutations for a more detailed analysis of the effects on glycerol transport: the shortest truncation (Fig. 1) that caused osmosensitivity (*FPS1*-Δ*11*, residues 217–230), the longest truncation that did not cause osmosensitivity (*FPS1*-Δ*9*, residues 13–215) and alanine mutations (Fig. 3) that caused different degrees of osmosensitivity: L225A, N228A, T231A, and P232A. We observed that the alleles that conferred osmosensitivity could less well retain the glycerol they produced, suggesting that they had a higher capacity of glycerol transmembrane flux (data not shown). To monitor this effect more directly, we determined the influx of radiolabelled glycerol following its concentration gradient in unstressed cells and during the first minute after shifting cells to 0.8 M NaCl. The different transformants displayed very different profiles of glycerol influx (Fig. 4; note that the two graphs have different scales). Truncation of the regulatory domain (*FPS1*-Δ*11*) as well as mutations of Thr-231 and Pro-232 caused the highest levels of glycerol influx while mutation of Leu-225 caused intermediately high glycerol influx. These data correlate well with the degree of sensitivity to 0.8 M NaCl of cells expressing the same alleles (Figs. 1 and 3). The simplest interpretation of those data is that the regulatory domain controls and restricts glycerol flux through Fps1p.

The influx of glycerol is significantly reduced after hyperosmotic shock, as reported previously (14, 15). Some reduction was even observed in *fps1*Δ cells and hence is independent of Fps1p. Furthermore, a significant hyperosmotic shock-induced reduction of glycerol influx was also observed in cells expressing hyperactive Fps1p alleles. However, glycerol influx after hyperosmotic shock is still much higher in these cells than in cells expressing wild type Fps1p. This suggests that even hyperactive Fps1p lacking the N-terminal regulatory domain retains an ability to reduce channel activity upon hyperosmotic shock albeit not sufficiently to support normal glycerol retention and growth in high osmolarity medium.

The Position of the Regulatory Domain Is Important for Function—Pro-236, which is the most proximal amino acid to TMD1 apparently important for channel control, is located only 20 residues from the first TMD. We therefore asked if the exact position of the regulatory domain was important for its function. To this end, we inserted TS, TSL, and TSLS behind Ile-242, thereby increasing the distance between the regulatory domain and TMD1 by 2, 3, and 4 amino acids (Fig. 5). Increasing the distance between the regulatory domain and TMD1 reduced its apparent functionality as it resulted in progressively stronger sensitivity to 0.8 M NaCl. We also deleted amino

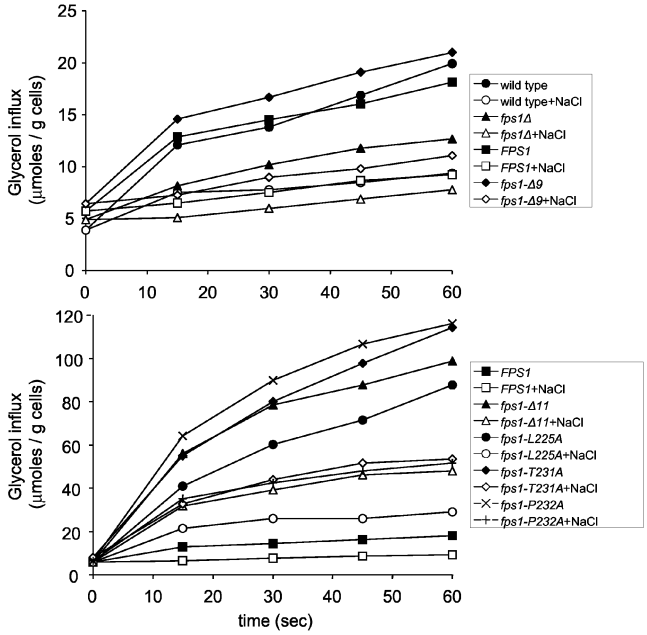

FIG. 4. **Glycerol influx.** Influx of glycerol into yeast *fps1*Δ cells expressing different *FPS1* alleles as a measure for Fps1p channel activity. Cells were incubated in the presence of a total of 100 mM glycerol containing 40 μM radiolabeled glycerol. *Error bars* indicating S.D. of three independent experiments are too short to display.

Allele	Sequence	0.8M NaCl
Wild type	235 LPSTYHPINKTSSV 248	
Plus 2 amino acids	235 LPSTYHPITSNKTSS 248	
Plus 3 amino acids	235 LPSTYHPITSLNKTSSV 248	
Plus 4 amino acids	235 LPSTYHPITSLSNKTSSV 248	
Minus 4 amino acids	235 LPST--TS--TSSV 248	
fps1-Δ11	Deletion of 217-230 (negative control)	

FIG. 5. **Moving the regulatory domain results in hyperactive channel.** The regulatory domain was moved 2, 3, and 4 amino acids further away from the first TMD or was moved four amino acids closer by deletion of four amino acids. Poor growth on 0.8 M NaCl indicates a hyperactive channel.

acids 239–244 and replaced them by threonine and serine thereby diminishing the distance by four amino acids. Also this manipulation resulted in osmosensitivity and hence poorer function of the regulatory domain (Fig. 5). All four *FPS1* alleles encoded functional glycerol export channels and were expressed in the plasma membrane (data not shown). Taken together, it appears that the exact position of the regulatory domain is important for restricting glycerol export.

The Regulatory Domain and Its Exact Position Are Conserved—We searched for sequence data of Fps1p orthologs from other yeasts in the *Saccharomyces* Genome data base SGD at genome-www.stanford.edu/Saccharomyces/, the Washington University Genome Center at www.genome.wustl.edu/blast/yeast_client.cgi and from the Genolevure data (45) at cbi.labri.fr/Genolevures/index.php. For a comparison we used the complete Fps1p sequences from *Saccharomyces bayanus* (90% identical to ScFps1p and of similar size; 661 *versus* 669 amino acids) and *Saccharomyces kluyveri* (56% identical, 647 amino acids) as well as partial sequences from *Kluyveromyces lactis* and *Kluyveromyces marxianus*. The *K. lactis* and *K. marxianus* protein fragments are 72% identical to each other and 46 and 42% identical, respectively, to Fps1p from *S. cerevisiae*.

There is high sequence identity over the six TMDs and four of the five loops (Fig. 6 and data not shown). Loop A is much longer in the yeast glycerol facilitators as compared with that of the bacterial GlpFs (19), and it seems to be poorly conserved among the different yeast proteins. We note that KlFps1p, KmFps1p, and SkFps1p have the typical MIP family NPA motifs in loop B, whereas both ScFps1p and SbFps1p have NLA. In loop E, three version of the NPA motif exist: NPA (KlFps1p), NLA (ScFps1 and SbFps1), and NMA (SkFps1p). Proximal to TMD1, the regulatory domain identified in this work is highly conserved and represents, together with TMD1 and loop B, the most highly conserved segments. Also the distance between the regulatory domain and TMD1 is conserved. Proximal of the regulatory domain there is little apparent sequence conservation except for about 25 amino acids around position 110 (ScFps1p; not shown); the function of this domain is not known.

The Regulatory Domain Is Predicted to Have Structural Similarity to Loops B and E and May Dip into the Membrane—Surprisingly, the N-terminal regulatory domain of Fps1p exhibits significant structural similarity to loops B and E of Fps1p and GlpF. Those loops dip from both sides into the membrane forming two bell-shaped half TMDs, which, together with genuine TMDs, form the channel and its central constriction (22, 25, 28).

The sequence alignment (Fig. 7*A*) centers around conserved NP motifs (NL in Fps1p loop E). The loop E sequences both have a methionine residue 9 amino acids before the NP motif, which is also found in the regulatory domain (here called loop N). There is also a threonine residue conserved (although not positionally) on the approach to the NP motif. Two amino acids before NP the loop B sequences have a histidine, and the regulatory domain has a tyrosine in that position; both residues are bulky and polar. Furthermore, threonine residues occupy similar positions after the NP motif in both the loop B and loop N sequences. Threonine is frequently involved in hydrogen bond interactions between transmembrane regions.

There are two main requirements that a loop dipping into the membrane bilayer must satisfy. Firstly, it must be sufficiently amphiphilic to allow it to enter the membrane, but not go all the way through. The NP motif is inherently hydrophilic and for this reason there must be a series of relatively hydrophobic amino acids at the beginning and the end of the dipping domain to allow the central section to remain in the membrane. Secondly, it must contain amino acids that allow it to adopt a suitable conformation, usually a β-turn that is sufficiently hydrophobic to enter the membrane and a strong α-helix to ensure the positioning of the NP motif and a spatially precise exit.

In order to test those characteristics for the regulatory domain we employed a range of prediction methods. Using the Kyte and Doolittle scale and Eisenberg scale (in parentheses), the average hydrophobicity of the regulatory domain is −0.52 (0.103). This is within the range of those of the B and E loops of Fps1p (0.667 (0.333) and −0.75 (0.01) respectively), and GlpF (1.1 (0.5) and −0.224 (0.221) respectively). Therefore the regulatory domain appears sufficiently hydrophobic to reside in the membrane.

We then analyzed the propensity for α and β conformation of loops B and E and the regulatory domain. The Deleage and Roux (Fig. 7*B*), and also Levitt scales (not shown) indicate a strong α propensity (and a weak β propensity) immediately preceding the NP motif. This is in line with the predictions for membrane dipping loops B and E and in accordance with the known structure of GlpF (25).

We have modeled the region around the regulatory domain (Val-216–Trp-245), using the GlpF dipping loop B (Ile-56–Lys-85) as a template of known structure (Fig. 7*C*). The majority of the regulatory domain comodels with GlpF loop B but some differences in conformation are indicated. In the regulatory

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K. lactis       SR--STTGPN-----QNSQ---TAAXPNXPSNVNGAVTMMVKPKTXYQNPQTPTVLPSTX 65
K. marxianus    SP--DPALQN-----QNNEGGVPANDPNDPNNVNNAITMMVKPKTLYQNPQTPTVLPSTY 140
S. kluyveri     AAPMQPRVSNDIVSVNSDSAGRGGSDENDPHIHN--VPMMVKPKTLYQNPQTPTVLPSTY 177
S. bayanus      RSRAESNTGHSATTGATNGRTTGAQTNMENNEPPRSVPIMVKPKTLYQNPQTPTVLPSTY 230
S. cerevisiae   RSRATSNAGHSANTGATNGRTTGAQTNMESNESPRNVPIMVKPKTLYQNPQTPTVLPSTY 239
                     .   :      ..     .              :.:****** ************
                                          TMD1                        loop A
K. lactis       YPXNKWSSFKYQHMKEFFGEXLGTMIMMMFGTAVNCQRKLSQQNQINKFNQIIQLNNMES 125
K. marxianus    YPINKWSSFKYQHMKEFFGEFLGTMIMMMFGTAVVCQSKLSEQDKINQFNQILAMNHKSN 200
S. kluyveri     HPINKWSTVKYNYLKEFLAEFIGTMMMIIFGSAVVCQVRLSQQQQKNIFSQKLIEANLTN 237
S. bayanus      HPINKWSSVKNTYLKEFLAEFMGTMVMIIFGSAVVCQVNVAGKIQQDNFNSALDNIKVTD 290
S. cerevisiae   HPINKWSSVKNTYLKEFLAEFMGTMVMIIFGSAVVCQVNVAGKIQQDNFNVALDNLNVTG 299
                :* ****:.*  ::***:.* :***:*::**:** ** .:: : : : *.  :   :  .
                                                TMD2
K. lactis       X------QIAMLQYLATPDVAGNFATVAFGWAAAVVMGYFAAGGSAISGAHLNPAITVSN 179
K. marxianus    D------DISMLQYIATPNVAGNFVSIAFGWAGAVVMGYFAAGGSAISGAHLNPAITVSN 254
S. kluyveri     S------DVSMLQYLVTPDPGGNFDNIALGWAGAVVMGYFSAGGSAISGAHLNPALTLSN 291
S. bayanus      SSAETIETMKSLTSLVSSVAGGTFDDVALGWAAAVVMGYFCAGGSAISGAHLNPSITLAN 350
S. cerevisiae   SSAETIDAMKSLTSLVSSVAGGTFDDVALGWAAAVVMGYFCAGGSAISGAHLNPSITLAN 359
                     :  *  :.:.  .*.*  :*:***.*******.*************::*::*
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FIG. 6. **Sequence comparison.** The predicted sequence of *S. cerevisiae* Fps1p is compared with those from *S. bayanus, S. kluyveri*, *K. lactis,* and *K. marxianus*. *X* stands for any amino acid that could not be predicted due to DNA sequence ambiguity. An *asterisk* indicates where all sequences are identical, a *colon* where the sequence is conserved. Only the sequences around the regulatory domain (*black with white letters*) and the transmembrane domains (*TMDs*) 1 and 2 (*gray*) as well as loops A and B (with the *underlined* NPA motif) are shown.

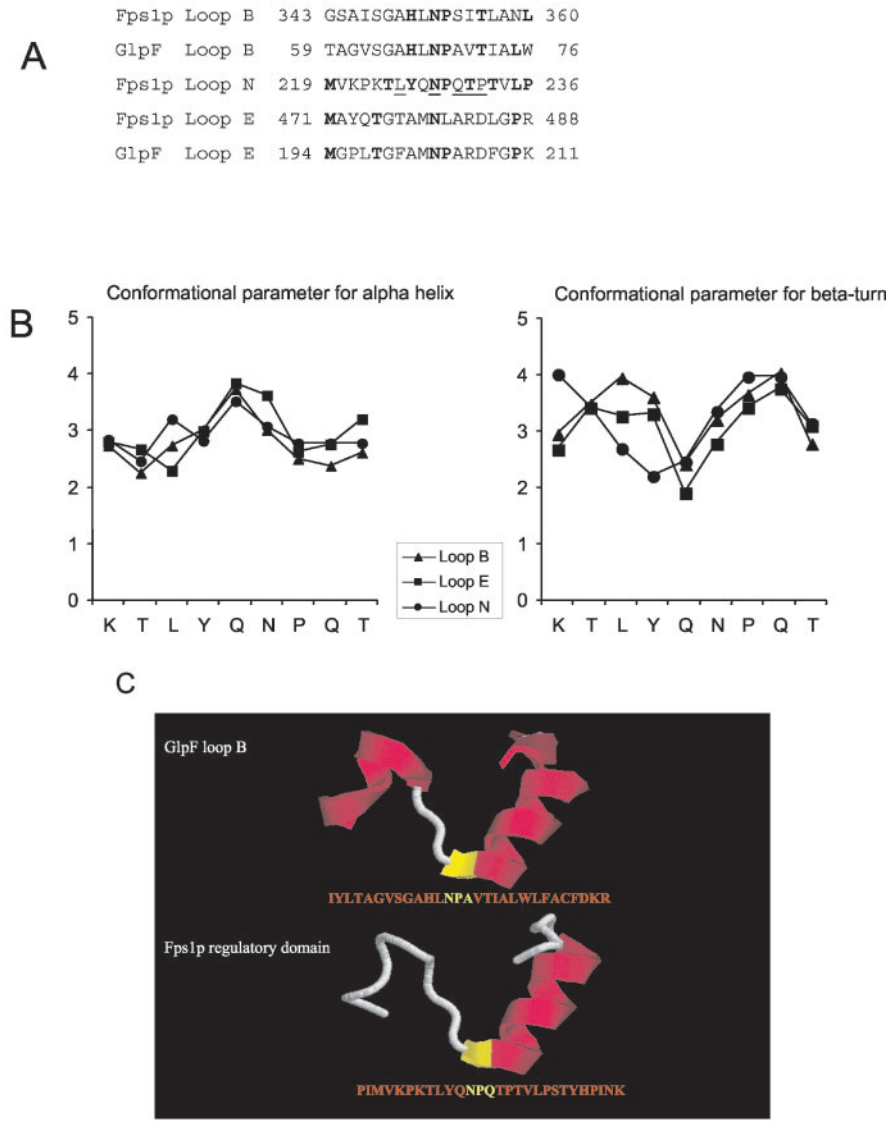

FIG. 7. **The regulatory domain shows similarity to loops B and E from GlpF and Fps1p.** *A*, alignment of the regulatory domain of Fps1p and loops B and E from GlpF and Fps1p. Conserved residues discussed in the text are presented in *bold*, and residues shown to be particularly sensitive to exchange against alanine are *underlined*. *B*, Deleage and Roux plots of conformational parameters for α-helix and β-turn conformations for the N-terminal regulatory domain and averaged loops B and E from Fps1p and GlpF. *C*, model of the structure of the N-terminal regulatory domain of Fps1p based on x-ray crystallography data of GlpF loop B. The constituent amino acids are shown beneath each loop.

loop N, the β structure before the motif is more extensive, due to the disruption of any preceding α structure by the frequent proline residues. Similarly, the α structure following the NP motif runs for only 2.5 turns, compared with 4 turns for GlpF loop B. This fits with the predicted extramembrane location of the region between the regulatory domain and TMD1. In contrast loop B of GlpF is preparing to reenter the membrane at Lys-85 for TMD3. Interestingly, according to this model the residues shown to be particularly important for channel control, Thr-231 and Pro-232, are on the same side of the helix as

Asn-228 and likely to be involved in stabilizing hydrogen bond interactions with that residue.

Intragenic Suppressors Confirm that the Hyperosmosensitive Phenotype of Fps1p Truncations Is Due to Hyperactive Transport—Truncation or mutation of the N-terminal regulatory domain caused poor growth on high osmolarity plates (Figs. 1–3). In an attempt to obtain more information on the structural basis of Fps1p regulation we isolated and characterized suppressors of this osmosensitive phenotype. We chose for the suppressor screen the two shortest deletions within the N terminus that gave such a phenotype. *Fps1*Δ mutant cells expressing *FPS1-Δ10* or *FPS1-Δ11* were plated on selective medium (SC; lacking leucine for plasmid selection) containing 0.8 M NaCl and spontaneous osmoresistant colonies were obtained at a frequency of about 1–2 $\times$ 10^{-5}. We reasoned that intragenic suppressors should either restore channel control or abolish transport function. In order to eliminate mutants in which Fps1p was fully inactivated we tested for survival of a hypo-osmotic shock. Indeed, the majority of the osmoresistant clones carried *FPS1* alleles that proved non-functional in this test (data not shown). We obtained 13 mutant clones out of an initial 33 selected that conferred both resistance to high osmolarity while retaining the ability to complement the hypo-osmosensitivity of the *fps1*Δ mutant. The 13 clones corresponded to just four point mutations probably because they were clonal repeats. Those mutants carried exchanges of A410E, S429F, A469P, and R483H. All alleles were expressed at similar levels (not shown), they conferred almost wild type resistance to high osmolarity (Fig. 8*A*) and accumulated the glycerol they produced like wild type cells (data not shown). The four mutant alleles complemented the *fps1*Δ mutant for tolerance to a hypo-osmotic shock to different degree (Fig. 8*B*). Transformation with wild type *FPS1* and allele S429F resulted in an about 100-fold higher proportion of cells surviving a hypo-osmotic shock as observed with control *fps1*Δ cells. Alleles A410E, A469P, and R483H conferred an intermediate sensitivity, *i.e.* increased the proportion of surviving cells by about a factor of ten. These observations correlated well with the glycerol export capacity within the first 5 min after a hypo-osmotic shock. While wild type Fps1p mediated the release of 65% of the intracellular glycerol, cells lacking Fps1p released less than 5% of their glycerol. The S429F allele conferred the highest glycerol release of the four mutants (26%). The other three alleles conferred low level of glycerol export (around 13%; A410E, about 19%), which, however, was consistently higher than that observed in *fps1*Δ cells. These low levels are apparently just sufficient to allow a 10-fold increase in survival as compared with control cells lacking Fps1p.

Hence, the observed suppression of the sensitivity to increased osmolarity conferred by the truncated *FPS1* alleles is probably due to a strongly reduced glycerol export rate. Interestingly, all four mutations mapped to regions of Fps1p facing to the outside of the cell and were located in loops C and E and in TMDs 4 and 5 (Fig. 8*C*). Ala-410 is conserved in *E. coli* GlpF and replaced in the Fps1p orthologs by small hydrophilic residues; hence glutamic acid may affect the structure of this loop. The position of Ser-429 at the beginning of TMD5 is commonly taken by small amino acids and hence the more bulky phenylalanine may disturb this helix. Ala-469 is conserved in GlpF and the *Kluyveromyces* Fps1p orthologs and the change to proline is expected to alter secondary structure of loop E, which is crucial for channel function. Arg-483 is located immediately distal to the important NPA (NLA) motif. Its replacement in human AQP2 renders this protein non-functional and causes a pathological phenotype (46).

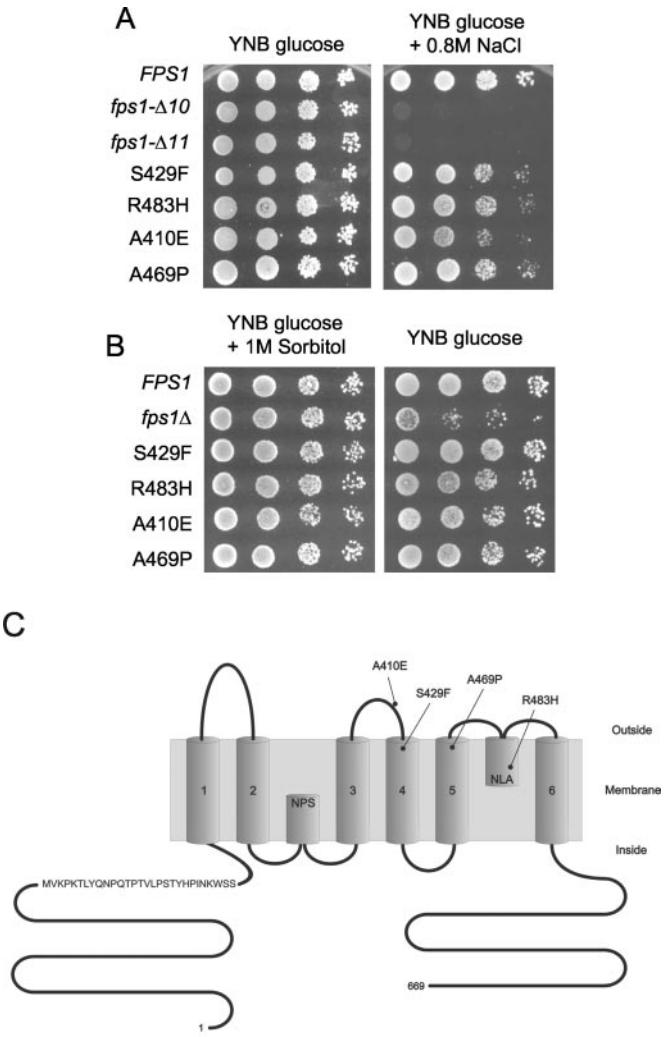

FIG. 8. **Intragenic suppressor mutations of the osmosensitivity conferred by hyperactive Fps1p.** *A*, growth phenotypes on 0.8 M NaCl of *fps1*Δ mutants expressing the four different mutant alleles. The ability to grow in the presence of salt indicates suppression of the osmosensitive phenotype of *fps1-Δ10* or *fps1-Δ11*. *B*, growth phenotypes of the same set of strains after shift from 1 M sorbitol to medium without sorbitol. The ability to survive and normally grow after such an osmotic downshift indicates presence of a functional channel; the *fps1*Δ mutant is sensitive to such conditions. *C*, sketch of the Fps1 protein showing the six TMDs, the NPS and NLA motifs in loops B and E, respectively, the regulatory domain loop N identified in this work, and the four mutations that suppress the sensitivity to high osmolarity conferred by truncation of the regulatory domain.

DISCUSSION

In this work we have defined a short segment in the N-terminal extension of Fps1p that is required for restricting glycerol transport through that MIP channel. Taking deletion and mutagenesis analysis as well as sequence comparison together, we conclude that the regulatory domain is located between residues 219 and 239 with its core between 225 and 236.

The role of this regulatory domain is to restrict the transport through Fps1p. There are several lines of evidence that support this notion. First, yeast cells expressing Fps1p that lacks the regulatory domain or carries specific mutations in this domain show a strongly enhanced glycerol transport both under standard conditions as well as under high osmolarity (Refs. 14–16, 31, and this work). Second, cells expressing such *FPS1* alleles adjust poorly to high osmolarity conditions, overproduce glycerol and leak glycerol into the growth medium (Refs. 14–16, 31, and this work). Finally, mutations that suppress the sensitivity

to high osmolarity of cells expressing hyperactive Fps1p map to *FPS1* and mediate strongly reduced flux through Fps1p. This observation directly demonstrates that hyperactive glycerol transport through Fps1p is the cause of the observed osmosensitivity.

The regulatory domain seems to perform its restricting function both under standard conditions as well as under high osmolarity; truncation or mutation of the domain causes higher glycerol uptake both in the presence and the absence of hyperosmotic stress (Fig. 4). We have previously shown that glycerol flux through the yeast plasma membrane is rapidly regulated by altered osmolarity: it is decreased upon a shift to high osmolarity to ensure internal accumulation of glycerol produced by the cell and it is increased upon a hypo-osmotic shock to allow rapid glycerol export (14, 15). Part of the reduction of glycerol flux upon a hyperosmotic shock is independent of Fps1p, as is also observed in cells lacking *FPS1* (Fig. 4). This could be due to other transport proteins or passive diffusion through the lipid bilayer, which could be affected by altered membrane tension. It also appears that the Fps1p-dependent glycerol flux through the hyperactive channel is partially diminished upon hyperosmotic shock (see for instance *fps1-Δ11* in Fig. 4) but remains at a much higher level than that observed for wild type Fps1p. While these data are in line with the notion that Fps1p is controlled by gating, it appears that the regulatory domain studied here is not the only determinant for a possible gating mechanism. It rather seems as if the regulatory loop N keeps Fps1p in a conformation that allows it to readily restrict glycerol transport both in the presence and absence of osmotic stress. In the absence of this domain transmembrane glycerol flux is too high to allow efficient accumulation of glycerol produced by the cell and hence sensitivity to high osmolarity.

One way to interpret these observations is that the proposed gating mechanism operates in a stepwise manner as has been shown for the mechanosensitive MscL channel from bacteria. MscL, which mediates unspecific solute export upon severe hypo-osmotic shock, seems to exist in a closed, extended and an open state dependent on the degree of cell swelling (8–10). While the physiological roles of Fps1p and MscL appear to be similar there is no evidence that the mechanisms underlying their control are similar too. However, based on the analysis of the role of the N-terminal regulatory domain one might speculate that the regulatory loop N plays a role in the transition from one state to another, such as between a fully open state and an expanded state. Such a stepwise opening/closing mechanism may make sense given the various roles Fps1p plays in yeast cells: it is not only needed for bulk, rapid glycerol export upon hypo-osmotic shock but also for moderate, sustained glycerol export during growth under anaerobic conditions (15) and for turgor control during cell fusion of mating yeast cells (47).

Unexpectedly, the regulatory domain is predicted to exhibit structural similarity to the channel forming loops B and E of Fps1p and GlpF and has the potential to form a loop that dips into the membrane; loops B and E have been shown to do so based on structural analysis of GlpF and AQP1 (22, 25, 28). With the regulatory loop N, the four amino acids preceding the motif are of similar hydrophobicity to loops B and E (due in large part to Leu-225) and would be well suited to enter the membrane. In this regard, it is interesting that Leu-225 and Tyr-226 seem to be critical as their simultaneous mutation to TS abolished channel control. Replacement of Leu-225 by alanine also affects the function of the regulatory domain (Fig. 3). Position 225 being very close to the membrane face could be critical for membrane insertion, and it is feasible that this is affected even by replacement with alanine. This is particularly salient in light of the hydrophilicity of the amino acids surrounding Leu-225 on each side (KT and YQ), rendering the section dependent on a highly hydrophobic residue at its center in order to maintain a sufficiently hydrophobic nature for membrane insertion. Mutation of Tyr-226 to the more hydrophobic phenylalanine has no obvious effect (Figs. 2 and 3).

How could such a membrane dipping domain control channel function? In one possible scenario it may assist in orienting the transmembrane domains and dipping loops B and E into a conformation that diminishes glycerol flux (such as in an expanded, partly open conformation). In that case it could be amendable to changes in membrane conformation upon osmotic changes and participate in a gating mechanism by altering the relative orientation of the TMDs and dipping loops involved in transport. While such a mechanism might be analogous to that for MscL it should be noted that there are limitations to the comparison between Fps1p and MscL. While MscL probably was designed as a gated efflux channel Fps1p is part of a large family of channel proteins where only AQP6 has been reported to be controlled by gating (23), although not by osmotic changes; hence Fps1p may have adopted a control mechanism as a secondary event in evolution.

In a different scenario the regulatory domain could directly interact with loop B and hence with the transmembrane pore. Such direct interactions would require the spacing between the regulatory loop N and the TMDs to be particularly important, as we have shown here to be the case. Some observations from structural modeling could also support such an idea. If membrane inserted, the intramembrane portion of the N loop is likely to be shorter than those of loops B and E, starting from around Thr-224. This raises the possibility that the N loop might lie buried in the bilayer close to the extramembrane face, with either of the glutamine residues possibly in interaction with His-350 of the B loop. This could bring the two NP motifs of loops B and N into close proximity. Perhaps the regulatory loop N pushes against the pore, thereby altering its open/closed probability. Upon membrane stretch such pushing may be increased or decreased thereby either increasing the open or the closed probability. However, more work is needed to understand the underlying mechanisms. Crucial aspects of ongoing work encompass further mutational studies and the purification of the protein such that it can be reconstituted for *in vitro* studies and to open the possibility for structural analyses.

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A Short Regulatory Domain Restricts Glycerol Transport through Yeast Fps1p

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