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Troy Beeler
Uniformed Services University of the Health Sciences

Kenneth Gable
Uniformed Services University of the Health Sciences

Chun Zhao
Uniformed Services University of the Health Sciences

Teresa Dunn
Uniformed Services University of the Health Sciences

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A Novel Protein, CSG2p, Is Required for Ca^{2+} Regulation in *Saccharomyces cerevisiae**

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Troy Beeler, Kenneth Gable, Chun Zhao, and Teresa Dunn‡

From the Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Nineteen mutants that lost the ability to grow in 100 mM Ca^{2+} (but remained insensitive to 50 mM Sr^{2+}) were identified in a screen of approximately 60,000 mutagenized yeast colonies. Cells carrying mutations in the *CSG2* gene grow normally in low Ca^{2+} medium but have decreased growth rates when the Ca^{2+} concentration is above 10 mM. The *csg2* mutant cells accumulate much higher levels of Ca^{2+} in a compartment that is exchangeable with extracellular Ca^{2+} but the nonexchangeable Ca^{2+} pool which predominates in wild-type cells is not influenced. Sr^{2+} influx is not increased in the *csg2* mutant cells. Mg^{2+} decreases the amount of Ca^{2+} in the nonexchangeable pool without influencing the *csg2*-induced exchangeable Ca^{2+} pool. The data indicate that the *csg2* mutation causes a selective increase in Ca^{2+} accumulation into a pool which is distinct from the vacuolar pool. The *CSG2* protein consists of 410 amino acids, contains nine putative transmembrane segments, four potential sites for *N*-linked glycosylation, and a sequence with homology to the EF-hand Ca^{2+}-binding site.

It has been difficult to demonstrate that *Saccharomyces cerevisiae* requires Ca^{2+} for vegetative growth using Ca^{2+}-depleted rich media (YPD-Ca^{2+}) or Ca^{2+}-free synthetic media. Nonetheless, results from numerous lines of investigation indicate that Ca^{2+} plays an important regulatory role in *S. cerevisiae* (1, 2). Many proteins known to be regulated (directly or indirectly) by Ca^{2+} are present in *S. cerevisiae* including calmodulin (3), calmodulin-dependent protein kinase (4), calmodulin-regulated protein phosphatase (5), kex2 protease (6), trehalase (7), glycogen phosphorylase (8), phospholipase C (9), vacuolar K^+ channel (10), and protein kinase C (11). Furthermore, numerous Ca^{2+}-sensitive (12–14) and Ca^{2+}-dependent (15, 16) mutants have been isolated.

Since *S. cerevisiae* has become an experimental model system for investigating the many eukaryotic processes that are controlled by Ca^{2+}, (3, 17) it is of interest to identify the genes and proteins that are important in regulating the cytosolic Ca^{2+} concentration of *S. cerevisiae*. The resting cytosolic Ca^{2+} concentration (100–200 nM (17, 18)) is apparently maintained by the active transport of Ca^{2+} from the cytosol into internal organelles such as the vacuole, and out of the cell through the plasma membrane (19–21). For Ca^{2+} to serve as a regulatory signal, mechanisms must also exist for Ca^{2+} to flow into the cytosol in response to the appropriate stimuli.

One approach toward identifying the genes (and the proteins) which are important in regulating the cytosolic Ca^{2+} is to isolate mutants that lose the ability to grow in medium containing high Ca^{2+} concentrations, identify those mutants with alterations in the regulation of cellular Ca^{2+}, and then clone and characterize the genes which complement the mutations. Anraku and co-workers (13) have generated a collection of Ca^{2+}-sensitive mutants that represent 18 complementation groups. Nine of the genes are required for vacuolar morphogenesis or are subunits of the vacuolar proton ATPase (14). One of the genes is allelic to *CDC24* (22).

We have recently generated a collection of Ca^{2+}-sensitive mutants using a screening protocol similar to that of Anraku and co-workers (13) except that the mutagenesis and prescreen growth was done in media containing 1 mM EGTA and the primary screen was done at 37 °C to allow identification of temperature-sensitive, Ca^{2+}-sensitive mutants. Because defects in the vacuolar transport system are expected to cause Sr^{2+} sensitivity, we focused on the mutants that are specifically sensitive to Ca^{2+} but resistant to Sr^{2+} hoping to identify candidates for novel Ca^{2+} transport genes not related to vacuole function. Here we report on our collection of *csg* (Calcium Sensitive Growth) mutants and the molecular/biochemical analysis of one of the isolates, *csg2*, which has identified a novel protein that may regulate Ca^{2+} accumulation by a non-vacuole organelle.

EXPERIMENTAL PROCEDURES

Materials—Arsenazo III was obtained from Aldrich. $^{45}Ca^{2+}$ was purchased from DuPont-New England Nuclear. Zymolyase 100T was supplied by Seikagaku Kogyo, Rockville, MD. All other chemicals were purchased from Sigma.

Strains, Growth Media, and Transformations—The strains used in this study are DBY947(*α ade2–101 ura3–52*), TDY2003 (*α ade2–101 ura3–52 csg2–1*), TDY2040 (*α ade2–101 trp1Δ leu2Δ ura3–52 csg2::LEU2*$^+$), and TDY821 *mata/α ura3–52/ura3–52 lys2/+ +/ade2–101 trp1Δ/trp1Δ leu2Δ/leu2Δ can*^{R}*/can*^{S} or were derived from them by standard genetic crosses. Yeast media for growth and sporulation were prepared according to Sherman *et al.*(23). Yeast were transformed by the method of Ito (24) except that 5 μg of sonicated salmon sperm DNA was routinely added to each reaction.

Bacterial strains SCS1, JM109, and JM110 were used for the propagation of plasmids and M13 phage. M13mp18 and mp19 phage and the pRS316 phagemid (25) were used to generate single-stranded template DNA for sequence determination.

Mutagenesis—DBY947 or an isogenic *a*-mating strain was mutagenized by a modification of the method described by Lindegren *et al.* (26). Cells (10^6/ml) in YPD + 1 mM EGTA were treated with 1.0% EMS[1] for 90 min at 26 °C which induced about 70% killing. The cells were diluted 1000-fold into YPD + 1 mM EGTA and allowed to recover for 4 h. Aliquots (200 μl) were removed and spread on YPD agar plates contain-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L24113.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Tel.: 301-295-3592; Fax: 301-295-3512.

[1] The abbreviations used are: EMS, ethylmethanesulfonate; PIPES, 1,4-piperazinebis(ethanesulfonic acid); ORF, open reading frame.

ing EGTA. A metal-pronged replicator was used to transfer cells to plates containing different medium designed to find Ca^{2+}-related phenotypes. Colonies that scored as mutants in the primary screen were retested in a second screen and finally tested by streaking for single colonies.

Nucleic Acid Manipulation—DNA was prepared from yeast by the method of Holm *et al.* (27) Plasmid DNA was prepared from *Escherichia coli* by the method of Holmes and Quigley (28). Sequence determination was accomplished with the ABI 373A DNA Sequenator using the primer sequencing kit of ABI for the M13mp18 and mp19 templates. The entire sequence was determined for both strands.

CSG2 Gene Cloning—The *CSG2* gene was cloned by complementation of the Ca^{2+}-sensitive phenotype of the *csg2-1* mutant. Strain TDY2003 was transformed with a YCp50-based genomic library (29), selecting first for uracil prototrophs and subsequently replica plating to YPD + 100 mM $CaCl_2$ plates. When the Ca^{2+}-resistant transformants were grown on YPD to allow plasmid segregation, the resulting *ura*$^-$ segregants (selected on 5-fluoroorotic acid plates (30)) simultaneously reverted back to the Ca^{2+}-sensitive phenotype. The plasmid, pB2-1, contains an insert common to all the complementing plasmids (Fig. 1).

To demonstrate linkage of the cloned DNA to the *CSG2* locus, we constructed an integrating plasmid by subcloning a 2000-base pair *Hin*dIII-*Sal*I fragment from the yeast DNA insert of pB2-1 into YIp5. The resultant plasmid was used to integratively transform a wild-type (DBY947) strain thereby marking the chromosomal locus homologous to the insert of pB2-1 with the *URA*$^+$ gene. A *URA*$^+$ transformant having the YIp5 plasmid integrated at the appropriate locus (confirmed by Southern blot analysis (31)) was mated to a *csg2-1* haploid, the resulting diploid was sporulated, and tetrads were dissected. In all 16 tetrads analyzed, parental ditype segregation of the Ca^{2+}-sensitive and URA$^+$ phenotype was observed indicating that the cloned fragment includes the wild-type *CSG2* gene.

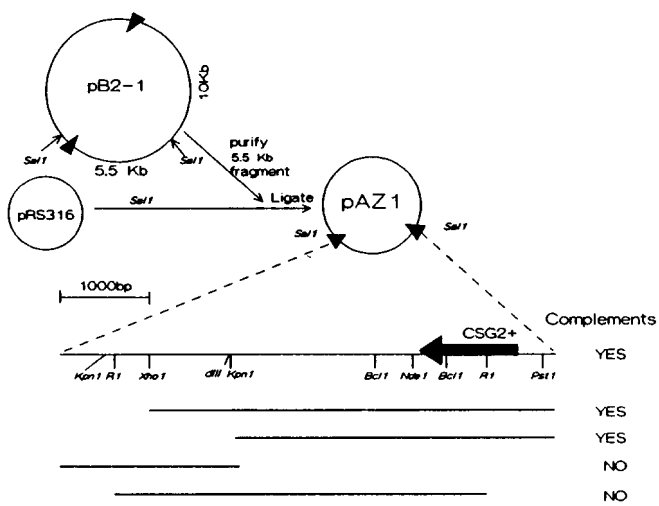

FIG. 1. **Localization of the *CSG2* gene on the complementing plasmid and restriction map of pAZ1.**

Localizing the CSG2 Gene—To localize the *CSG2* gene on the complementing plasmid, fragments of the cloned yeast DNA were subcloned. A single *Sal*I site within the insert separates the cloned yeast DNA into 5.5- and 10-kilobase fragments; subcloning of the 5.5-kilobase fragment into pRS316 localized the *CSG2* gene to the smaller *Sal1* fragment (Fig. 1). The complementing activity was further delineated by subcloning restriction fragments into pRS316 and testing for their ability to confer growth on high Ca^{2+}.

Construction of the CSG2 Null Allele—A null allele of *CSG2* in which about 800 base pairs of *CSG2* coding sequence was replaced with the *LEU2*$^+$ gene was generated by inserting the *Kpn*I to *Pst*I fragment carrying *CSG2* (Fig. 1) into pRS306; this plasmid was then linearized with *Eco*RI, incubated with *Bal31* to remove about 800 base pairs, and a *Xho*I linker was ligated in at the deletion junction. A *Sal*I fragment carrying the *LEU2*$^+$ gene was ligated into the *Xho*I site and the *Kpn*I to *Pst*I fragment of this plasmid was used to transform the diploid strain TDY821 to *LEU2*$^+$ (32). *CSG2*$^+$/*csg2*::*LEU2*$^+$ heterozygotes were identified by Southern blot analysis (31).

Ca^{2+} Accumulation by Ca^{2+}-sensitive Mutants—To identify mutants in the Ca^{2+}-sensitive collection likely to have a defective Ca^{2+} transport system, the amount of Ca^{2+} accumulated by cells grown in 10 mM Ca^{2+} was determined. Ca^{2+} loading of cells was measured by incubating cells in YPD at 37 °C for 1 h to induce any temperature-sensitive defect followed by a 1.5-h incubation in 10 mM Ca^{2+}. The cells were harvested and washed by centrifugation, and the amount of Ca^{2+} stored in the cells was determined spectrophotometrically using the Ca^{2+} indicator arsenazo III as previously described (18).

Measurement of Ca^{2+} Accumulation by Permeabilized Cells—Cells (10^9 cells/ml) were permeabilized by treatment with 0.2 mg digitonin/ml for 10 min at 27 °C followed by a 100-fold dilution into the calcium uptake medium. Ca^{2+} uptake was measured spectrophotometrically as previously described (18).

RESULTS

To identify Ca^{2+}-sensitive mutants, 60,000 EMS-mutagenized cells (approximately equal numbers of *a* and *α*-mating) were screened for the inability to grow at 37 °C on YPD plates containing 100 mM Ca^{2+}. The mutants were divided into two classes according to whether they are sensitive only to Ca^{2+} or whether they are sensitive to both 50 mM Ca^{2+} and 50 mM Sr^{2+}. Secondary biochemical screens were performed on the Ca^{2+}-sensitive mutants to determine their cellular Ca^{2+} loading after incubation in 10 mM Ca^{2+} for 1.5 h at 37 °C and the capacity for vacuolar Ca^{2+} transport by digitonin-permeabilized cells in the presence of 27 μM Ca^{2+} and 1 mM ATP.

Eighteen of the Ca^{2+}-sensitive mutants out of the total of 64 were able to grow in 50 mM Sr^{2+}. Complementation analysis of

TABLE I
Complementation analysis of CSG mutants

Diploids were tested for growth on 100 mM Ca^{2+} at 37 °C. Failure of the diploid to grow (–) indicates that both haploids have a mutation in the same gene.

		csg1				*csg2*					
		2000	2001	2002	2036	2003	2041	2020	2018	2022	2042
	2005	–	–	–	–	+	+	+	+	+	+
csg1	2006	–	–	–	–	+	+	+	+	+	+
	2031	–	–	–	–	+	+	+	+	+	+
	2035	+	+	+	+	–	–	+	+	+	+
csg2	2010b	+	+	+	+	–	–	+	+	+	+
	2004	+	+	+	+	+	+	+	+	+	+
	2008	+	+	+	+	+	+	+	+	+	+
	2026	+	+	+	+	+	+	+	+	+	+
	2032	+	+	+	+	+	+	+	+	+	+

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-309                                                                GCCTGCAGA
-300  GCAGCGCGCCAAGCAGAAGGAGGCATGGTACTCCTTCTTATTCAAATAAGATTGTAAATATATGCTACTTTCTTC
-225  CTTGTATATACATATATACTTATAACGGTATAACCCGTTCTTGTAATATCGGCTATCACCCGGCGAAGGTGTATC
-150  GCAAGAAAAAAAAAAAGCTCTCTCACTCTAAAGGAGGCTATGTGAACGTGGGAAGATCAAGTGAAAGAAATGTTTG
-75   TAGGCCATTTTCTTCCAGAACAGATCCGCTCTTGAGCTGGTGAGTTAGCACGATAACAAACAAAGATACAGCGTC

+1    ATGTCTACCACACTACTTTGGTTTTCAAGTGTAATAGGCTACGTGATTCAAACAAAATGTTTGTCTAACATACAA
       M  S  T  T  L  L  W  F  S  S  V  I  G  Y  V  I  Q  T  K  C  L  S  N  I  Q    +25

+76   TCTAAAAAGGAAATCTCCGTGGGGCCCAATGGTACAATTGCAACGCCTGAAACTAACGGCGACAACGGAAACTCA
       S  K  K  E  I  S  V  G  P  N  G  T  I  A  T  P  E  T  N  G  D  N  G  N  S    +50

+101  AGTTCATTAACCTTCTATCTGACCTTTATGTATTTTGCTTCGTGGCTGCTCTGGGTGCCTGCATCTCGACTTTGG
       S  S  L  T  F  Y  L  T  F  M  Y  F  A  S  W  L  L  W  V  P  A  S  R  L  W    +75

+176  GAGAAGATGAGACCGATGTTTGTCTCTGACTCAGACTCGAACAGGAATTCTCAGTTTGACAACAACAACAGCGGG
       E  K  M  R  P  M  F  V  S  D  S  D  S  N  R  N  S  Q  F  D  N  N  N  S  G    +100

+251  TCTGTGACAAACGAAGATGTCGATACGTTCTCGCACGTGTTGGATGATCCTCAACCACGGATTCCAGCCCAACAG
       S  V  T  N  E  D  V  D  T  F  S  H  V  L  D  D  P  Q  P  R  I  P  A  Q  Q    +125

+326  CAGAAGCAAAAAATCATATCCGTGGCTACCTTCAAATATGTGGCTAAGCTAACAGTGCTGGCTCTCATAATGATT
       Q  K  Q  K  I  I  S  V  A  T  F  K  Y  V  A  K  L  T  V  L  A  L  I  M  I    +150

+401  GTCGCTGATTTGACTTATAACATGGCTTTGTCATTGTCACCGGCATTTGATGTTGCTTTGATGCAAAATACTGCC
       V  A  D  L  T  Y  N  M  A  L  S  L  S  P  A  F  D  V  A  L  M  Q  N  T  A    +175

+476  ATTTTCGAAATTGTCACTTTACTATATGGTGTTTGTGGAATCTCCAGGAAGAACTACGTTTTCCGTAATTTCCTC
       I  F  E  I  V  T  L  L  Y  G  V  C  G  I  S  R  K  N  Y  V  F  R  N  F  L    +200

+551  ATCATGATGAACGCGGTCATTGGAATTTTGATCATCTCATACACGAAGGCTACCTGTGACATGCTTGCCGGAAAG
       I  M  M  N  A  V  I  G  I  L  I  I  S  Y  T  K  A  T  C  D  M  L  A  G  K    +225

+626  CTGTCCGTCAACCCTAACACGGGTGAACTTTCTGACCCATTCTTGTTTGATAGGTTGAAAGGTGCTCTGATTTGC
       L  S  V  N  P  N  T  G  E  L  S  D  P  F  L  F  D  R  L  K  G  A  L  I  C    +250

+701  GGCCTTGGTGCTTTGATTATGGGTCCTTTTGCCGTGTTATGGAACCGTTGGTTTTGCAGTAACATTTCCAAGAAC
       G  L  G  A  L  I  M  G  P  F  A  V  L  W  N  R  W  F  C  S  N  I  S  K  N    +275

+776  GAAAATTCTGCTGTAGTCTTGGTTAAGCAGAGCACCCACATGGCCCTAATCGGTATTATTGGCATGGTAATACTT
       E  N  S  A  V  V  L  V  K  Q  S  T  H  M  A  L  I  G  I  I  G  M  V  I  L    +300

+851  TTGCCATTTATTCCTAAATTTCCCTCCCGTGAGTCTGTGGAATCCATTTCGTTGTTCTATAATGACAAGAGCTTT
       L  P  F  I  P  K  F  P  S  R  E  S  V  E  S  I  S  L  F  Y  N  D  K  S  F    +325

+926  TGGTTCTCTCTACTAGGCTCGATTATCTTTGGTTCCTTGCCGAGCTTGATTTCGATATTAGAGTTGAATCGCAAG
       W  F  S  L  L  G  S  I  I  F  G  S  L  P  S  L  I  S  I  L  E  L  N  R  K    +350

+1001 GCCCCTGCTGAGTATTTGACGACGTGCAACCTGGGAGCTATTATCTTTATGGGGTTAGCTGAGTGGGTTTGCGAA
       A  P  A  E  Y  L  T  T  C  N  L  G  A  I  I  F  M  G  L  A  E  W  V  C  E    +375

+1076 CCTACGCAAACCACAATTGTGAGATGGGAAGTCATAGGATACATAATGCTAACGGTAAGTTTGTTGGTCCTATCA
       P  T  Q  T  T  I  V  R  W  E  V  I  G  Y  I  M  L  T  V  S  L  L  V  L  S    +400

+1151 GTAACACTCGGGGAAGGTAAATACCACCATTAGTACGGACAACATACTTTATATGACTGATGATGATGTAACAAG
       V  T  L  G  E  G  K  Y  H  H  U                                              +410

+1226 CATATGCAATTATTTGGTTTCCCTAAATAAAAAATCCCAATCAATATCTTGATTATTTTCAAGACAATTACTAGG
+1301 ATGTTCTTCTATTTTTTTATTTTTATTTGTATATTGTATATTCTAAAAAGAAGAAGCCATTTGGTGGGCTTTATT
+1376 ATAAATATTAAGAGGCATACCTCCGCCTATCCGCCTATCCTCGTCATGAACAATCAATTAAAAGCGCTTATAAAG
+1451 CGATATACAAG
```

FIG. 2. **Nucleotide and translated amino acid sequence of *CSG2*.** The sequence was determined as described under "Experimental Procedures." The sequence of a putative Ca^{2+}-binding site is *underlined*.

these *csg* mutants identifies two major complementation groups and eight independent isolates (Table I).

Among the Ca^{2+}-specific mutants, those in the *csg1* and *csg2* complementation groups have an average 315 and 1090% increase in the cellular Ca^{2+} relative to wild-type when incubated for 1.5 h in 10 mM Ca^{2+} at 37 °C. All other Ca^{2+}-specific mutant isolates show wild-type levels of Ca^{2+} accumulation except for the mutant strain TDY 2022 which shows a 680% increased Ca^{2+} accumulation.

Because mutants in the *csg2* complementation group show the most dramatic Ca^{2+} over-accumulation phenotype and have an extremely tight Ca^{2+}-sensitive growth phenotype, this group was characterized first. A heterozygous *csg2–1*/*CSG2*$^{+}$ diploid grows normally on YPD + 100 mM Ca^{2+} indicating that the mutation is recessive. Tetrad analysis of the heterozygous diploid shows 2 Ca^{2+}-sensitive:2 Ca^{2+}-resistant spore colonies for each 4-spored tetrad indicating that the mutant phenotype is due to a single nuclear mutation.

The wild-type *CSG2*$^{+}$ gene was cloned by selecting for its ability to complement the *csg2–1* Ca^{2+}-sensitive phenotype as described under "Experimental Procedures." The *CSG2* gene was localized within the complementing fragment of genomic DNA by subcloning various restriction fragments and testing their ability to complement the *csg2–1* Ca^{2+}-sensitive phenotype.

Nucleotide Sequence of CSG2—The sequence from the *Eco*RI site in both directions was determined. The *Eco*RI site was found to lie within a large open reading frame (ORF). The entire nucleotide and deduced amino acid sequence of the predicted 45,579-dalton protein is presented in Fig. 2.

A comparison of the *CSG2* ORF to sequences in the NBRF and EMBL GenBank databases shows that an amino-terminal portion of the *CSG2* ORF is identical to an unidentified ORF at the end of the cloned 1.7-kilobase DNA fragment that also contained the full *SCO1* gene. *SCO1* was mapped to chromosome II (33, 34).

The hydropathy analysis of *CSG2* indicates the existence of up to nine transmembrane sequences (Fig. 3*A*). A possible topological representation of CSG2p is shown in Fig. 3*B*. A potential signal sequence is found at the amino terminus. It contains a sequence of 12 hydrophobic amino acids followed by two potential signal peptidase cleavage sites (TKC ↓ LS ↓ N) (35, 36).

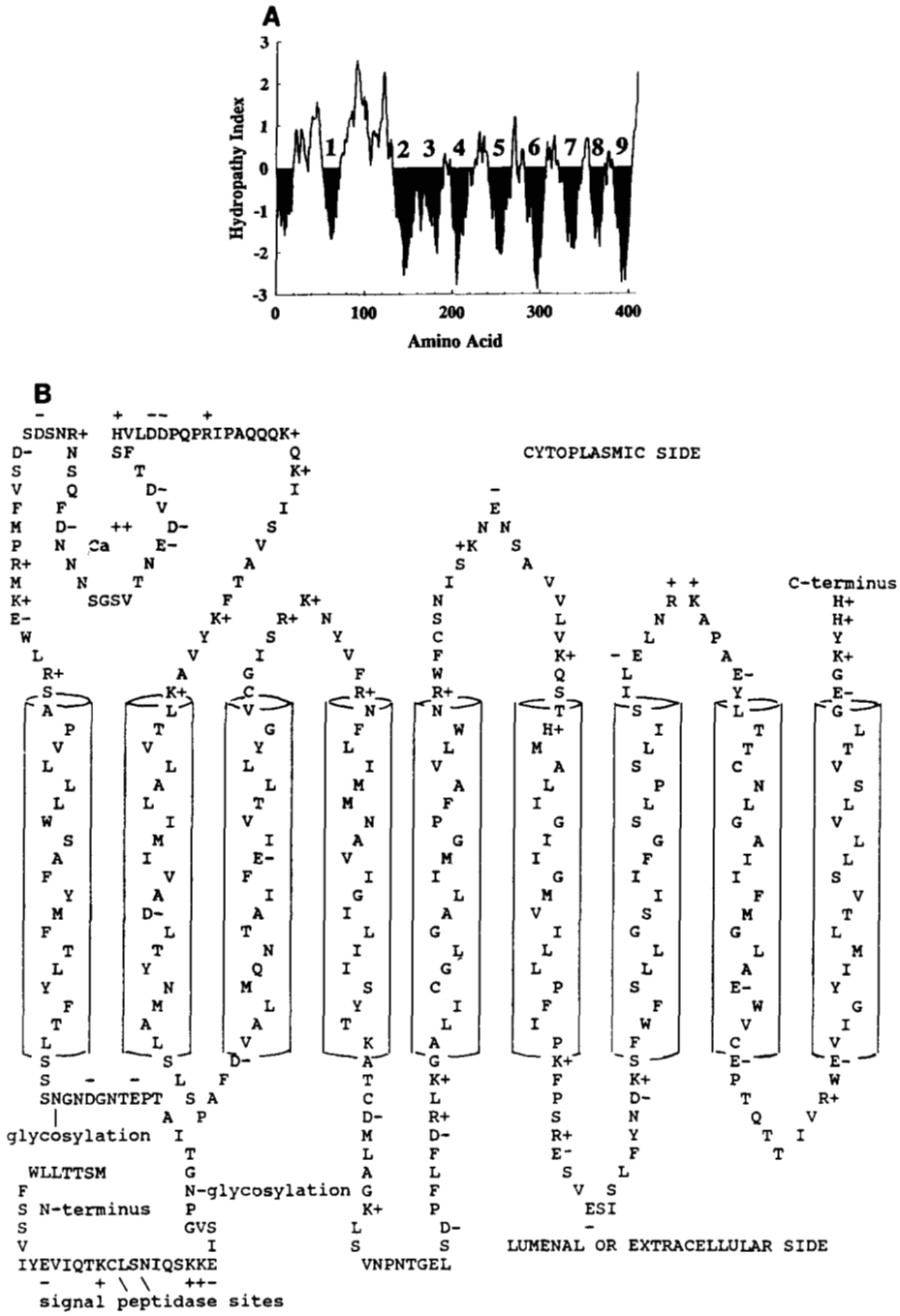

FIG. 3. **Identification of membrane spanning segments and potential glycosylation and Ca^{2+}-binding site on *CSG2p*.** *Panel A*, Kyte and Doolittle hydropathy plot of CSG2p. Each point on the curve represents a hydropathy average of a 14-amino-acid window centered at that point. Proposed transmembrane domains are *numbered 1–9*. *Panel B*, schematic representation of CSG2p showing possible signal peptidase cleavage sites, a putative Ca^{2+}-binding site, 2 *N*-linked glycosylation sites, and transmembrane segments.

Four consensus *N*-linked glycosylation sites are found; however, two of the four are placed on the cytoplasmic side of the membrane in our model suggesting that only two of the sites are available for glycosylation. A 12-amino-acid segment with homology to a consensus Ca^{2+}-binding loop of the EF-hand type is found in the first cytoplasmic loop (Fig. 3*B*). The comparison of this site to the consensus Ca^{2+}-binding site as determined by Marsden *et al.* (37, 38) is shown in Fig. 4.

Disruption of the Chromosomal CSG2 Locus—The four independent non-complementing alleles of *csg2* identified in our screen for Ca^{2+}-sensitive mutants all show similar biochemical phenotypes. To determine the phenotype of a null mutation at the *CSG2* locus, the cloned *CSG2* gene was used to construct a deletion/substitution at the *CSG2* locus as described under "Experimental Procedures." The disrupted allele, designated *csg2::LEU2*$^+$, was substituted at the chromosomal locus by a one-step gene replacement (32). Spore colonies having the *csg2::LEU2*$^+$ null allele are viable and grow comparably to wild-type on YPD medium but fail to grow on the same medium containing 50 mM Ca^{2+}. The null allele is similar to the EMS-derived *csg2* alleles with regard to growth properties and biochemical phenotypes. Hence it is the lack of functional CSG2p that causes Ca^{2+} sensitivity. The Ca^{2+} transport properties of the *csg2* null mutant are the same as those of the EMS-induced *csg2* mutants. Accordingly, all the biochemical data presented below is for the *csg2::LEU2*$^+$ null mutant strain.

Effect of Ca^{2+} on the Growth Rate of csg2 Mutant Cells—The rate of wild-type cell growth is influenced little by 50 mM Ca^{2+}. The growth rate of *csg2::LEU2*$^+$ cells in the absence of added Ca^{2+} is similar to that of wild-type, but in the presence of 50 mM Ca^{2+}, *csg2::LEU2*$^+$ cells fail to grow. Ca^{2+} concentrations as low as 10 mM decrease the growth of *csg2::LEU2*$^+$ at 26 °C (Fig. 5).

CSG2 Sequence 92-103										
Asp	Asn	Asn	Asn	Ser	Gly	Ser	Val	Thr	Asn	Glu	Asp
Amino acids that occur in the Ca^{2+}-binding loop of EF-hands with the percentage of total amino acids found											
Position in Ca^{2+}-Binding Loop											
1	2	3	4	5	6	7	8	9	10	11	12
ASP	Lys	Asp	Gly	Asp	**GLY**	Phe	Ile	Asp	Phe	**GLU**	Glu
98	28	73	51	56	89	16	70	33	16	31	86
Asn	Ala	**ASN**	Lys	**SER**	Asp	Tyr	**VAL**	Ser	Val	Asp	**ASP**
0.6	10	24	14	22	2	15	13	19	12	27	9
Tyr	Thr	Glu	**ASN**	Asn	Asn	Lys	Leu	Glu	Ala	Ala	Val
0.6	10	2.5	9	17	2	13	9	14	10	10	2
Glu	Gln	Ser	Arg	Gly	Lys	Thr	Met	**THR**	Glu	Lys	Asn
0.6	9	0.6	6	2	2	13	2.5	11	10	8	1
-	Val	-	Ala	Gln	Arg	Glu	Cys	Gly	Leu	Gln	Gln
-	8	-	5	0.6	1	6	2	10	10	5	0.6

FIG. 4. **Comparison of the amino acid sequence of a putative Ca^{2+}-binding site and the consensus Ca^{2+}-binding site of the EF hand.** The amino acids typed in *bold* are the ones found in CSG2p.

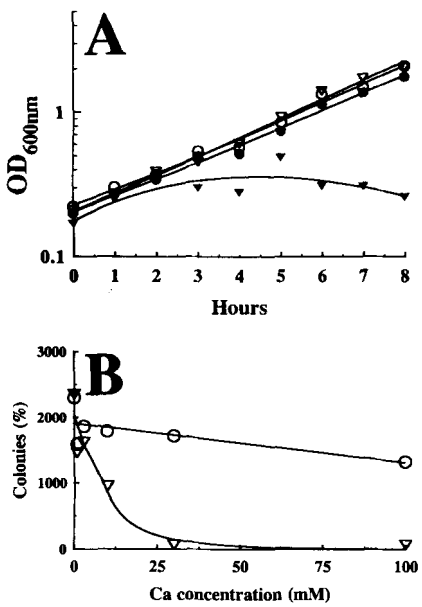

FIG. 5. **Effect of Ca^{2+} on the growth rate of wild-type and *csg2* mutant cells.** *Panel A*, wild-type (○, ●) and *csg2::LEU2*$^+$ (▽, ▼) cells were grown in YPD buffered to pH 4.7 with citrate at 26 °C to an OD_{600} of 0.2. The cells were then split to YPD, pH 4.7, with (●, ▼) or without (○, ▽) 50 mM $CaCl_2$. At the indicated time aliquots were removed, diluted 1:1 in 0.1 M K-EDTA, pH 7.6, and the OD_{600} was determined. Dilutions were made as necessary to maintain the OD_{600} between 0.1 and 0.3 throughout the experiment. *Panel B*, wild-type (○) and *csg2::LEU2*$^+$ (▽) cells were grown to an OD_{600} of 0.1 in YPD, pH 4.7, at 26 °C, split to six tubes, and 0, 1, 3, 10, 30, or 100 mM $CaCl_2$ was added. Aliquots were removed at 0 and 8 h, diluted, and spread on YPD plates to determine the number of viable cells. The number of cells at 8 h is plotted as a percent of the original cell number at $t = 0$.

Effect of csg2 Mutant on Cellular Ca^{2+} Levels—The rate as well as the amount of Ca^{2+} accumulated by *csg2::LEU2*$^+$ cells in 10 mM Ca^{2+} is greater than that of wild-type. Sr^{2+} accumulation by *csg2::LEU2*$^+$ cells was increased 2.5 times while Ca^{2+} accumulation increased 22 times indicating that Ca^{2+} accumulation was more influenced by the *csg2* mutation (Fig. 6).

In wild-type cells, most cellular Ca^{2+} is localized in the vacuole where it forms a stable, nonexchangeable complex with polyphosphate (18). To determine if the increased Ca^{2+} accumulated by *csg2::LEU2*$^+$ cells is also sequestered in a nonexchangeable Ca^{2+} pool, cells were incubated for 3 h in YPD medium containing 30 mM Ca^{2+} with 1 μCi/ml $^{45}Ca^{2+}$ and then shifted into YPD without $^{45}Ca^{2+}$ (Fig. 7).

For the *csg2::LEU2*$^+$ mutant the amount of nonexchangeable Ca^{2+} (~400 nmol Ca^{2+}/mg) is similar to that in wild-type, but the large increase in an exchangeable Ca^{2+} pool (~800 nmol

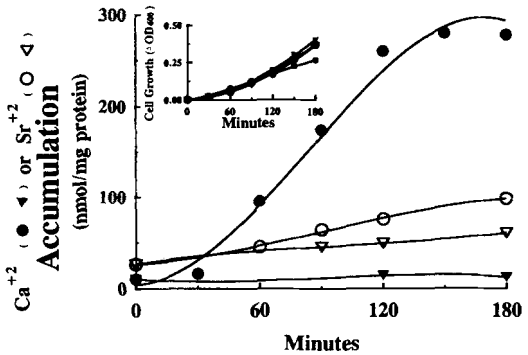

FIG. 6. **Ca^{2+} and Sr^{2+} accumulation by wild-type and *csg2* mutants cells.** Wild-type (▼, ▽) and *csg2* mutant cells (●, ○) were in exponential growth at 27 °C. At zero time, 10 mM Ca^{2+} (▼, ●) or Sr^{2+} (▽, ○) was added, and at various times aliquots were removed to determine viability and the amount of Ca^{2+} or Sr^{2+} accumulated. To determine the Ca^{2+} or Sr^{2+} loading level, cells were washed by centrifugation and resuspended to a density of 10^7/ml in KCl solution containing 50 μM arsenazo III. Accumulated Ca^{2+} or Sr^{2+} was released by digitonin (1 mg/ml) addition and measured spectrophotometrically by monitoring the difference absorbance of the arsenazo III-Ca^{2+} or arsenazo III-Sr^{2+} complex at 660–685 nm using an AMINCO-SLM dualbeam spectrophotometer. Viability (*inset*) was determined by diluting aliquots 10^4-fold and spreading 200 μl onto YPD plates.

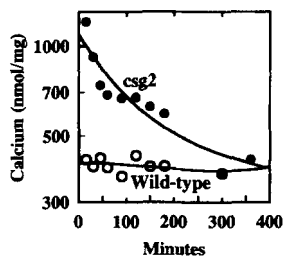

FIG. 7. **The *csg2*-induced Ca^{2+} pool is exchangeable.** Wild-type (○) and *csg2::LEU2*$^+$ (●) cells were grown to an $OD_{600\ nm}$ of 0.5 in YPD and diluted into YPD + 30 mM Ca^{2+}(1 μCi/ml) to a $OD_{600\ nm}$ of 0.1. After a 3-h incubation at 26 °C, the cells were washed three times by centrifugation with cold YPD and resuspended in YPD without $^{45}Ca^{2+}$. At the indicated times, the $^{45}Ca^{2+}$ remaining in the cells was determined by filtering 5-ml aliquots, washing the filters with 3 x 5 ml of cold 20 mM Mg^{2+}, drying the filters, and then measuring the $^{45}Ca^{2+}$on the filter by scintillation counting. At 5, 6, and 18 h after $^{45}Ca^{2+}$ release was initiated, the wild-type and *csg2::LEU2*$^+$ cells had essentially the same amount of $^{45}Ca^{2+}$.

Ca^{2+}/mg) suggests Ca^{2+} accumulation by a nonvacuolar organelle.

Another distinction between the *csg2*-induced Ca^{2+} pool and the vacuolar Ca^{2+} pool is the response of Ca^{2+} loading to increasing the Mg^{2+} concentration of the growth medium. Ca^{2+} accumulation by wild-type cells is blocked by extracellular Mg^{2+} (Fig. 8). Similarly the nonexchangeable Ca^{2+} pool in the *csg2* mutant is eliminated by Mg^{2+}, but the exchangeable Ca^{2+} pool (Fig. 8, *inset*) is not affected by Mg^{2+}, further indicating that the *csg2* mutant has normal vacuolar Ca^{2+} accumulation which is blocked by Mg^{2+}, but increased accumulation into a non-vacuolar organelle which is not influenced by Mg^{2+}. Normal vacuolar Ca^{2+} transport in *csg2::LEU2*$^+$ cells is further indicated by the observation that the rate and amount of vacuolar Ca^{2+} accumulation by permeabilized cells and isolated vacuole membrane vesicles is the same for *csg2::LEU2*$^+$ mutant cells as for wild-type (data not shown).

DISCUSSION

The data indicate that CSG2p is involved in the regulation of Ca^{2+} accumulation by an organelle which sequesters Ca^{2+} in an exchangeable form. The cytosolic Ca^{2+} concentration in both

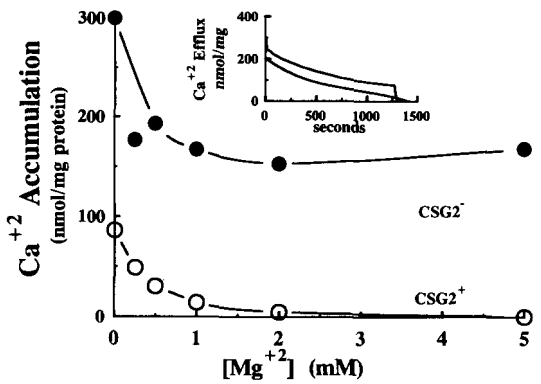

FIG. 8. **The effect of Mg^{2+} on the cellular Ca^{2+} content of wild-type and *csg2* mutants.** Wild-type (○) and *csg2::LEU2*$^+$ mutant (●) cells were grown to an OD_{600} of 1.2 and diluted 10-fold into YPD medium containing 50 mM $CaCl_{l2}$ and the indicated Mg^{2+} concentration (YPD medium has about 0.6 mM Mg^{2+}). After a 2-h incubation, the amount of Ca^{2+} accumulated was measured as described in Fig. 6. The *inset* shows the A23187-stimulated Ca^{2+}-release rate from *csg2::LEU2*$^+$ cells that were loaded in YPD (*top trace*) or YPD + 5 mM Mg^{2+} (*bottom trace*).

the wild-type and the *csg2::LEU2*$^+$ cells is apparently similar since vacuolar Ca^{2+} accumulation (polyphosphate dependent, Mg^{2+} inhibitable, and nonexchangeable) is not significantly influenced, and the cytosolic Ca^{2+} concentration as measured with fura 2 (data not shown) is not changed. The increased loading of *csg2::LEU2*$^+$ cells must, therefore, arise from altered Ca^{2+} fluxes across the membrane of the affected organelle.

The Ca^{2+}-induced killing and over-accumulation of Ca^{2+} by *csg2::LEU2*$^+$ cells is caused by the lack of CSG2 protein as opposed to an alteration in the properties of the protein. One model to explain the effect of the *csg2* mutation on cellular Ca^{2+} is that eliminating CSG2p directly causes an organelle to over-accumulate Ca^{2+}. If CSG2p directly mediates Ca^{2+} translocation across the organellar membrane, it must function in efflux. Alternatively, it might act to negatively regulate a Ca^{2+} pump. Because CSG2p is a membrane protein with a putative Ca^{2+}-binding site on the cytoplasmic side of the membrane, it is reasonable to suppose that the action of the protein may be regulated by cytosolic Ca^{2+}. The regulation of Ca^{2+} release from endoplasmic and sarcoplasmic reticulum by cytosolic Ca^{2+} (Ca^{2+}-induced Ca^{2+} release) has been demonstrated in other organisms. CSG2p is not homologous to the IP3-activated Ca^{2+} channel or the ryanodine-activated Ca^{2+} channel found in muscle cells; however, it might be a member of a new family of intracellular Ca^{2+} channel proteins. Although in wild-type cells, the amount of Ca^{2+} in the CSG2p-regulated Ca^{2+} pool is much less than that in the vacuole, it may still be important in regulating cytosolic Ca^{2+} levels in response to physiological signals since mobilization of only 1% of the cellular Ca^{2+} would raise the cytosolic Ca^{2+} concentration to 10 μM.

There are two alternative models which could explain the Ca^{2+} sensitivity of *csg2* mutants. 1) CSG2p is important in regulating a Ca^{2+}-dependent process, so that in the absence of functional CSG2p and in the presence of high Ca^{2+} concentrations, this Ca^{2+}-regulated process is inappropriately activated leading to cell death. 2) CSG2p mediates an important cellular process, for which another system can substitute in low Ca^{2+} concentrations; however, the alternative system is blocked by Ca^{2+} making CSG2p essential at high Ca^{2+} concentrations. Both models assume that the Ca^{2+}-overloading phenotype is a consequence of Ca^{2+}-induced cell damage. Although our data do not eliminate these two alternative models, we favor the model described above in which the *csg2* mutant directly causes an organelle to overaccumulate Ca^{2+} since specific Ca^{2+} accumulation by yeast cells is not generally associated with cell damage or death.

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