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yam8$^+$, a *Schizosaccharomyces pombe* Gene, Is a Potential Homologue of the *Saccharomyces cerevisiae MID1* Gene Encoding a Stretch-Activated Ca^{2+}-Permeable Channel

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The *Saccharomyces cerevisiae MID1* gene encodes a stretch-activated Ca^{2+}-permeable channel. In a protein database, we found a *Schizosaccharomyces pombe* gene whose predicted protein shows 26% identical and 62% similar to the Mid1 channel in amino acid sequence. cDNA derived from this gene, designated *yam8*$^+$, was isolated by reverse transcription-polymerase chain reaction (RT-PCR). Further analysis showed that the Yam8 protein consists of 486 amino acids and has 6 hydrophobic segments. The *yam8*$^+$ cDNA, placed under the *S. cerevisiae TDH3* promoter, partially complemented the mating pheromone-induced death (*mid*) phenotype of the *S. cerevisiae mid1* mutant. The expression of the *yam8*$^+$ cDNA in the *mid1* mutant cells partially remediated the *mid* phenotype and resulted in a slight increase in Ca^{2+} uptake activity. These findings suggest that Yam8 is a potential homologue of Mid1.

The *MID1* gene product (Mid1) is an N-glycosylated, integral membrane protein required for Ca^{2+} influx stimulated by mating pheromone (1). Cells lacking *MID1* die because of limited Ca^{2+} influx when incubated with mating pheromone. Electrophysiological and cell biological studies on Chinese hamster ovary (CHO) cells expressing Mid1 have revealed that it is a Ca^{2+}-permeable, stretch-activated nonselective cation channel (SA Cat channel) (2).

SA Cat channels are suggested to transduce mechanical strain into a cellular response, and thus play a crucial role in touch sensation, hearing, detecting gravity, and sensing osmotic changes (3, 4). Bacterial SA Cat channels are considerably studied because their genes and homologues are isolated, enabling ones to characterize them biochemically, electrophysiologically and structurally in conjunction with *in vitro* mutagenesis (5, 6). By contrast, such studies on eukaryotic SA Cat channels have just started after the finding of Mid1 as an SA Cat channel. Besides going into deeper analyses on Mid1, we need to find and analyze Mid1 homologues in other organisms for better understanding of eukaryotic SA Cat channels. Here we report the isolation and basic characterization of a potential *MID1* homologue, the *yam8*$^+$ cDNA, of the fission yeast *S. pombe.*

MATERIALS AND METHODS

Microbial strains and culture conditions. S. cerevisiae strains H207 (*MAT***a** *his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2*) and H301 (*MAT***a** *his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2 mid1-1*) was grown in rich medium (YPD) or synthetic media (SD and SD.Ca100). The compositions of these media were described previously (1). The *S. pombe* strain JY282 (*h*$^+$ *ura4*), provided by Dr. M. Yamamoto (University of Tokyo, Japan), was grown in YPD medium for the isolation of a cDNA clone of *yam8*$^+$. *Escherichia coli* strain DH5*α* was used as a host of plasmid DNAs.

Isolation of the S. pombe yam8$^+$ *cDNA and construction of a plasmid expressing the yam8*$^+$ *cDNA.* A *S. pombe* genomic clone (*yam8*$^+$, SPAC1F5.08C in Chromosome I, SWISS-PROT accession No. Q10063, GeneBank Accession No. Z68136) was identified as a potential *MID1* homologue through a BLAST search (7) on the *S. cerevisiae MID1* gene. To isolate the cDNA fragment encoding the *yam8*$^+$ ORF of *S. pombe,* RT-PCR was performed. Total RNA was extracted from *S. pombe* cells using YeaStar RNA extraction kit (Zymo Research, Orange, CA), and a poly (A)$^+$ RNA was isolated using Oligotex-dT30 Super (Takarashuzo, Kyoto, Japan). Total first-

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strand cDNA was synthesized using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ). PCR amplification was performed using a set of specific primers containing a restriction site: YAM8-Fwd/*Sal*I (5′-AGTGTCGACTAAAGGTTCT-AT**ATG**TTTTT-3′) and YAM8-Rv/*Not*I (5′-TATGCGGCCGCA**TTA**-CTCGACAAACAAAA-3′). The underlines show the *Sal*I and *Not*I sites, respectively, and boldface shows the initiation and termination codons, respectively. DNA polymerase used was *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and the template was the RT reaction products. After the initial denaturation at 94°C for 2 min, PCR amplification was carried out with 1 min at 94°C, 1 min at 48°C, and 3.5 min at 72°C for 25 cycles. The PCR-amplified DNA was digested with *Sal*I and *Not*I to obtain the 1,480-bp DNA fragment and the fragment was directly ligated under the promoter of a *S. cerevisiae* glyceraldehyde-3-dehydrogenase gene, *TDH3*, placed on the yeast expression vector pKT11 whose selection marker in yeast is the *URA3* gene. The resultant plasmid was designated pKT11-YAM8. DNA sequencing showed that the cloned cDNA has the correct nucleotide sequence (data not shown). To determine the nucleotide sequence, we employed the dideoxynucleotide chain-reaction method using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA) and ABI PRISM 377 DNA Sequencer (PE Applied Biosystems).

Transformation of S. cerevisiae cells. *S. cerevisiae* strains of H207 (*MID1 ura3*) and H301 (*mid1-1 ura3*) were transformed with pKT11-YAM8 and pKT11, according to the method of Ito *et al.* (8), with minor modifications. Ura^+ transformants were selected on plates containing SD medium supplemented with amino acids (20 μg/ml histidine, 30 μg/ml leucine and 20 μg/ml tryptophan) at 30°C for 3 days.

Determination of the viability of S. cerevisiae cells. The viability of cells was determined by the methylene blue liquid method described by Iida *et al.* (9).

Determination of Ca^{2+} uptake activity. We used two methods. For cells growing in SD.Ca100 medium, the method described by Iida *et al.* (1) was used. For cells suspended in buffer-glucose medium (10), the method described by Eilam and Chernichovsky (10) was generally followed with slight modifications.

RESULTS AND DISCUSSION

Identification of Yam8 as a potential Mid1 homologue. In order to find out Mid1 homologues in any organisms, we performed a BLAST search (7) by use of the amino acid sequence of the Mid1 protein and found a hypothetical 53.9-kDa protein C1F5.08C composed of 486 amino acid residues in chromosome I in *S. pombe.* This hypothetical protein has been designated Yam8 and its function has not been described in the literature. The CLUSTAL W multiple sequence alignment program (11) revealed that the Yam8 protein shares 26% amino acid sequence identity with Mid1 (Fig. 1). Although having the limited amino acid sequence identity, Yam8 shows significant sequence similarity (62%) to Mid1. Like Mid1, Yam8 has hydrophobic segments (up to six) and sixteen potential asparagine-linked (N-linked) glycosylation sites in the overall amino acid sequence, and two cysteine-rich regions in the C-terminal sequence. The cysteine-rich regions may be required for a putative protein–protein interaction. The *yam8*$^+$ gene has no intron.

The hydrophobic segments of Yam8 were designated H1 to H6 (Fig. 2A), among which the H2 and H4

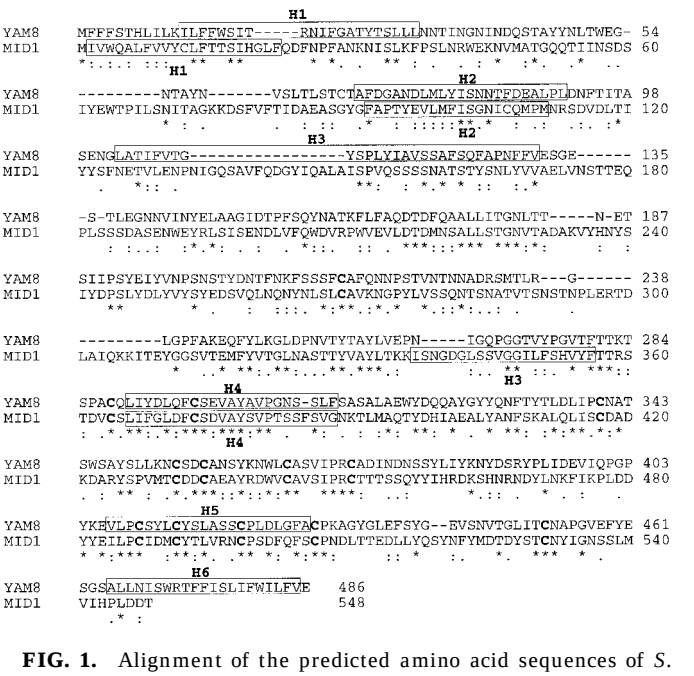

FIG. 1. Alignment of the predicted amino acid sequences of *S. pombe* Yam8 and *S. cerevisiae* Mid1. Asterisks indicate identical or conserved residues in all sequences in the alignment. Colons indicate conserved substitutions. Dots indicate semi-conserved substitutions. Boxes enclose the hydrophobic segments H1, H2, H3, H4, H5, and H6 of Yam8 and H1, H2, H3, and H4 of Mid1. The amino acid sequence of Mid1 was obtained from Ref. (1) (the Accession Number in EMBL/GenBank is D32133).

segments of Yam8 are similar to the H2 and H4 segments of Mid1, respectively, and the two segments have significant similarity to transmembrane segments of other ion channels (Figs. 2B and 2C). We also found amino acid sequences in three regions in Yam8 similar to some regions of other ion channels including Mid1, cGMP-gated channel or Ca^{2+} channel (Fig. 3), but functions of these sequences are unknown.

These cumulative structural features suggest that Yam8 is an ion channel whose function is similar to Mid1. It is of interest to note that the H2 and H4 segments of the Mid1 channel could serve as the channel wall of a putative tertiary structure of this channel (2). This structure is similar to the inward rectifier K^+ channel octagonal structure model (12). Thus, it is likely that the H2 and H4 segments of Yam8 also contribute to a similar structure, but it remains to be solved.

The yam8$^+$ *cDNA partially complements the mid phenotype.* To examine whether Yam8 of *S. pombe* is a functional homologue of Mid1, we cloned the *yam8*$^+$ cDNA by RT-PCR as described under Materials and Methods. The *yam8*$^+$ cDNA was then placed under the control of the *S. cerevisiae TDH3* promoter on the vector pKT11 and the resulting plasmid pKT11-YAM8 was introduced into the *mid1* mutant of *S. cerevisiae* (strain H301), which shows the mating pheromone-

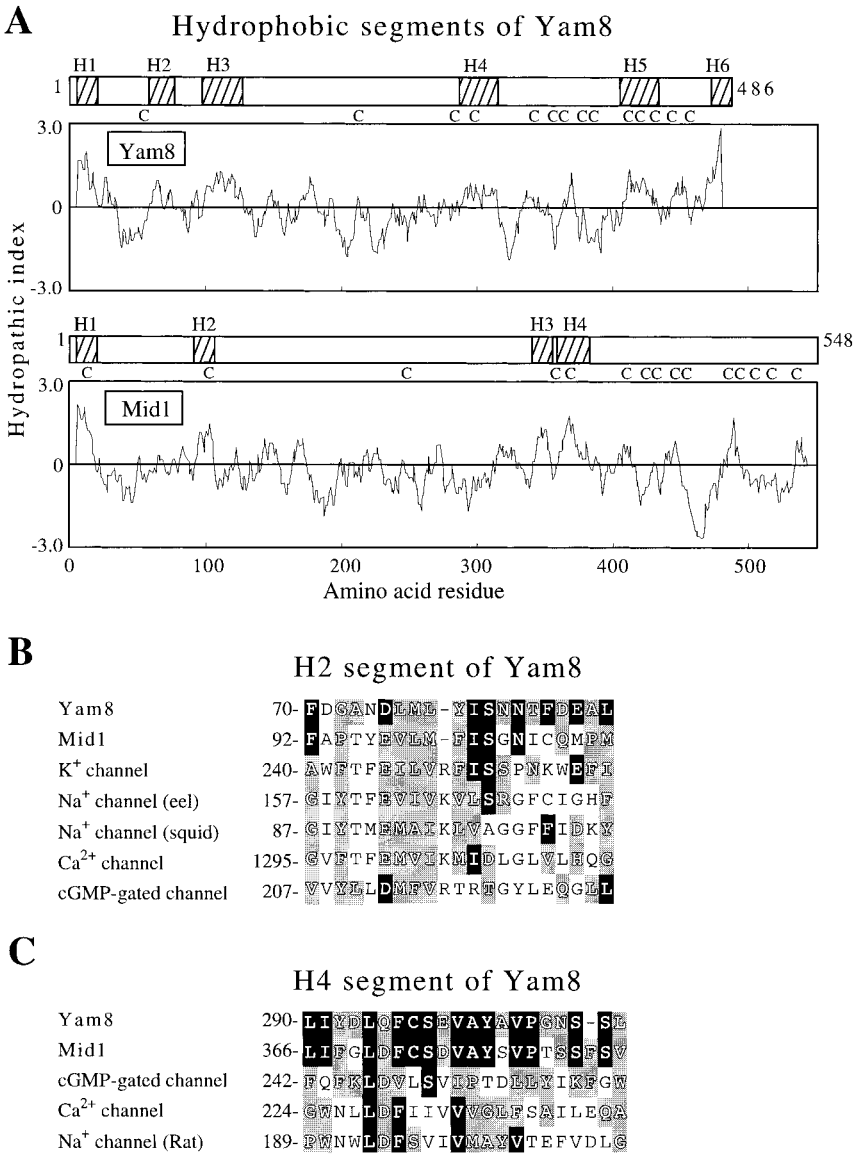

FIG. 2. Characteristics of Yam8. (A) Schematic diagram and hydropathy profile of Yam8 in comparison with those of Mid1. The hydrophobic regions are indicated by hatched boxes. Cysteine residues are indicated by "C" character. Hydropathy profiles are predicted by the Kyte and Doolittle algorithm (14). The range to average is 12. (B and C) Multiple sequence alignments of the H2 and H4 segment of Yam8 and other ion channels. Typical examples are shown here: The H2 segment of Mid1 (1), the H2 segment of *Drosophila* K^+ channel (15), the S2 segment of the first repeat of the eel Na^+ channel (16), the S2 segment of the first repeat of squid Na^+ channel (17), the S2 segment of the third repeat of rabbit brain Ca^{2+} channel (18), the H2 segment of bovine retinal cGMP-gated channel (19), and the S3 segment of the rat brain sodium channel type III (20). Identical and chemically similar residues are represented by highlights on black and gray backgrounds, respectively.

induced death phenotype (*mid* phenotype) and has a low Ca^{2+}-uptake activity (1). For control experiments, the vector pKT11 was also introduced into H301 and a wild-type strain, H207.

We noted that H301/pKT11-YAM8 grew slower than H301/pKT11. The mean generation times of H301/pKT11-YAM8 was 5.3 h, while that of H301/pKT11 was 2.5 h. This observation suggests that the *S. pombe* Yam8 protein has an inhibitory effect on cell proliferation in *S. cerevisiae* cells when expressed under the control of the *TDH3* promoter. To examine whether Yam8 complements the *mid* phenotype of the *mid1* mutant, the mating pheromone α-factor was added to cells of each strain growing in SD.Ca100 medium, after which the viability of the cells were determined. Because the mean generation time of each strain was different one another as mentioned above, the viability was presented as a function of generation after the addition of mating pheromone. Figure 4 shows that pKT11-YAM8 partially complemented the *mid* pheno-

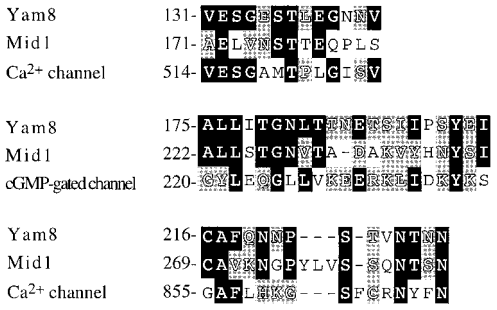

FIG. 3. Comparative alignments of Yam8 and other ion channels, cGMP-gated channel (19), and Ca^{2+} channel (21). Identical and chemically similar residues are represented by highlights on black and gray backgrounds, respectively.

type of the *mid1* mutant. Although its complementing activity was small, the results were reproducible. Thus it is likely that Yam8 is a functional homologue of Mid1.

Since another phenotype of the *mid1* mutant is a low Ca^{2+} uptake activity (1), we examined if this phenotype is also complemented by Yam8. $^{45}CaCl_2$ was added with or without α-factor to cells growing in SD.Ca100 medium, and the aliquots of each sample were filtered to determine Ca^{2+} accumulation at 0, 30, 60, 90, and 120 min after the addition. Ca^{2+} accumulation was plotted against generation of each strain. Figure 5 shows that Ca^{2+} accumulation in H301/pKT11-YAM8 cells is slightly greater than that in H301/pKT11 cells in the presence and absence of α-factor, although much smaller than that in wild-type H207/pKT11 cells.

This low complementing activity of pKT11-YAM8 was also confirmed by another method, which uses non-steady-state conditions for Ca^{2+} influx and is reported to show the rate of Ca^{2+} influx which is not affected by accumulation into the vacuole at least 30–40 sec after the start of the experiments (10). Exponentially growing cells were harvested, washed with Mes/Tris buffer containing glucose and no added Ca^{2+}, resuspended in the same solution, incubated for 90 min and then subjected to the measurement of Ca^{2+} uptake. The uptake was started by the addition of $CaCl_2$ labeled with $^{45}Ca^{2+}$. Because the 'zero-time' measurements represent $^{45}Ca^{2+}$ binding to the cells, they were subtracted from $^{45}Ca^{2+}$ transport data to show values for Ca^{2+} influx across the plasma membrane, as recommended by Eilam and Chernichovsky (10). In these experimental conditions, biphasic pattern of Ca^{2+} uptake can bee seen. The first is a fast component, which becomes saturated after 30–40 s, and the second is a linear increase in Ca^{2+} accumulation into the vacuole about 3 min after the start of the experiments. Figures 6A and 6B show that in 1 min after the addition of $^{45}CaCl_2$, the rate of calcium influx was slightly greater in H301/pKT11-YAM8 cells than in H301/pKT11 cells. However, 1 min later, the rate appeared to be essentially the same between the two strains. The Ca^{2+} uptake after 1 min may represent accumulation into the vacuole (10).

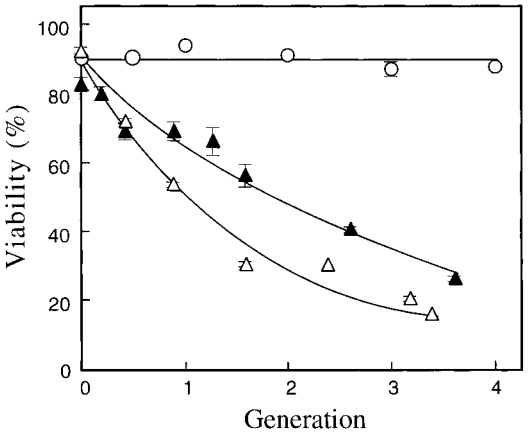

FIG. 4. Partial complementation of the *mid1* mutation by *yam8*$^+$. Exponentially growing cells of the strains H207 (wild-type)/pKT11, H301 (*mid1*)/pKT11 and H301 (*mid1*)/pKT11-YAM8 received 6 μM α-factor, and the cell viability was determined at the times indicated by the methylene blue liquid method (9). ○, H207/pKT11; △, H301/pKT11; ▲, H301/pKT11-YAM8. Vertical bars represent SE.

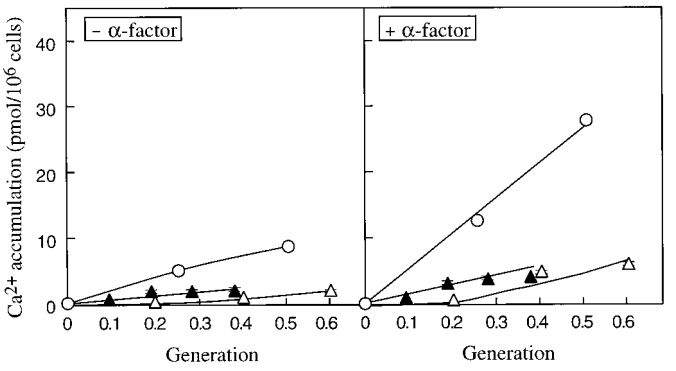

FIG. 5. Calcium accumulation in *mid1* cells transformed by *yam8*$^+$. Exponentially growing cells cultured in SD.Ca100 medium were incubated with $^{45}CaCl_2$ (185 kBq/ml; 1.85 kBq/nmol) in the presence (right panel) or absence (left panel) of 6 μM α-factor. At the times indicated, aliquots were taken, filtered through Millipore filters (type HA; 0.45 μm) that had been presoaked in 5 mM $CaCl_2$ and washed five times with the same solution. The radioactivity retained on the filters was counted by a liquid scintillation counter. Each point presented was the average of results from two independent experiments. ○, H207 (wild-type)/pKT11; △, H301 (*mid1*)/pKT11; ▲, H301 (*mid1*)/pKT11-YAM8. Vertical bars represent SE.

In our experimental conditions with the yeast strains we used, the biphasic patterns were not so obvious in our data as in reported ones (10). Although we do not know the reason, we believe that our data shows a partial complementing activity of Yam8 in the *mid1* mutant.

We recently learned that a mutant allele of the *yam8*$^+$ gene, designated *ehs1-1*, had been identified and characterized in *S. pombe*. The *ehs1* mutants have

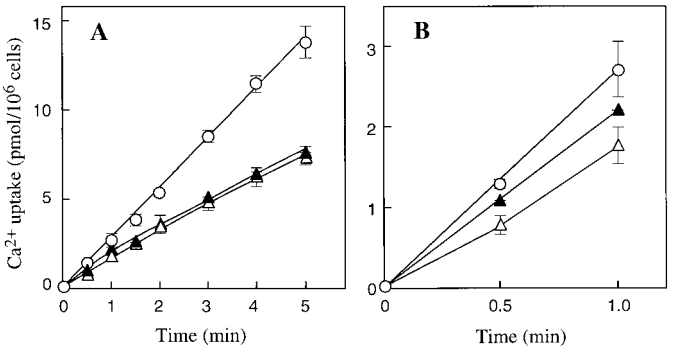

FIG. 6. Calcium uptake activity in *mid1* cells transformed by *yam8*$^{+}$. (A) Cells grown in SD.Ca100 medium were preincubated at 10^{-8} cells/ml in Mes/Tris buffer (10 mM, pH 6.0) containing 100 mM glucose and 1 μM $CaCl_2$ for 90 min at 30°C (10). Calcium uptake was initiated by adding $^{45}CaCl_2$ solution to a final concentration of 72 kBq/ml (0.72 kBq/μmol). Each point represented were the average of results from two independent experiments. (B) A part of the graph in (A) was enlarged, so that the differences in the initial rate of Ca^{2+} uptake among the three strains are clearly seen. ○, H207 (wild-type)/pKT11; △, H301 (*mid1*)/pKT11; ▲, H301 (*mid1*)/pKT11-YAM8. Vertical bars represent SE.

low Ca^{2+} uptake activity and display mating defect, like the *mid1* mutants of *S. cerevisiae*, indicating that Yam8/Ehs1 is really required for Ca^{2+} influx and mating in its own cells (Y. Sanchez, personal communication). This finding supports the idea that Yam8/Ehs1 is a structural and functional homologue of Mid1.

In summary, the present study indicated that Yam8 is a potential homologue of Mid1. The partial complementing activities shown by viability and Ca^{2+} uptake might be due to the limited similarity in overall amino acid sequence between Mid1 and Yam8. Although the overall amino acid similarity is small, some particular portions are well conserved. These portions could serve as probes for detecting other potential Mid1 homologues from other organisms, especially from higher eukaryotes, because the divergence of homologous genes between *S. pombe* and *S. cerevisiae* is similar to that between yeasts and mammals (13).

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