Essential Hydrophilic Carboxyl-terminal Regions Including Cysteine Residues of the Yeast Stretch-activated Calcium-permeable Channel Mid1*

The yeast *Saccharomyces cerevisiae* MID1 gene encodes a stretch-activated Ca$^{2+}$-permeable nonselective cation channel composed of 548 amino acid residues. A physiological role of the Mid1 channel is known to maintain the viability of yeast cells exposed to mating pheromone, but its structural basis remains to be clarified. To solve this problem, we identified the mutation sites of *mid1* mutant alleles generated by *in vivo* ethyl methanesulfonate mutagenesis and found that two *mid1* alleles have nonsense mutations at the codon for Trp$^{441}$, generating a truncated Mid1 protein lacking two-thirds of the intracellular carboxyl-terminal region from Asn$^{389}$ to Thr$^{548}$. *In vitro* random mutagenesis with hydroxylamine also showed that the carboxyl-terminal region is essential. To identify the functional portion of the carboxyl-terminal domain in detail, we performed a progressive carboxyl-terminal truncation followed by functional analyses and found that the truncated protein produced from the *mid1* allele bearing the amber mutation at the codon for Phe$^{422}$ (F522Am) complemented the mating pheromone-induced death phenotype of the *mid1* mutant and increased its Ca$^{2+}$ uptake activity to a wild-type level, whereas N521Am did not. This result indicates that the carboxyl-terminal domain spanning from Asn$^{389}$ to Asn$^{521}$ is required for Mid1 function. Interestingly, this domain is cysteine-rich, and alanine-scanning mutagenesis revealed that seven out of 10 cysteine residues are unexchangeable. These results clearly indicate that the carboxyl-terminal domain including the cysteine residues is important for Mid1 function.

The molecular mechanisms by which mechanical signals direct biological responses remain a frontier in the field of signal transduction. Electrophysiological studies have indicated that mechanotransduction can be mediated by ion channels that open or close in response to mechanical stimuli (1–5). Such channels play essential roles in a wide variety of activities including cell volume control, development, morphogenesis, and neuronal signaling underlying touch, hearing, and balance. However, eukaryotic mechanosensitive ion channels have not been cloned until recently, and thus little is understood of their structures and functions at the molecular level. Recently, genes encoding eukaryotic mechanosensitive channels or their candidates have been found in some species, including *MID1* in budding yeast (6, 7), *MEC4* in the nematode (8, 9), *SIC* in the rat (10), and *NOMPC* in the fly (11). *A MID1* homologue in fission yeast, *ehs1*/*yam8*, has been reported (12, 13).

Ca$^{2+}$ signaling constitutes an important backbone pathway, which is essential for a wide variety of cellular events. In *Saccharomyces cerevisiae*, Ca$^{2+}$ has essential roles in the mating process induced by the mating pheromone, α-factor (14). This process is divided into early and late stages, and Ca$^{2+}$ seems to be required for the late stage only (15). In the early stage of this process, the mating pheromone binds to its cell surface receptor coupled with a heterotrimeric GTP-binding protein that activates a mitogen-activated protein kinase pathway, and the cells are eventually arrested in the G$_1$ phase of the cell cycle (16). In the late stage, at about 30 min after the binding of the mating pheromone, the cells differentiate into morphologically distinct cells having a mating projection, calledshmoo, in which the cell wall and plasma membrane should be rearranged to support the polarized growth. This change accompanies Ca$^{2+}$ influx necessary for the viability of shmoo (14). When incubated in Ca$^{2+}$-deficient medium, wild-type cells can grow normally in the absence of the mating pheromone but die after differentiation into shmoo if the mating pheromone is added (14).

Mutants showing the mid (mating pheromone-induced death) phenotype have been isolated, one of which is the *mid1* mutant (17). This mutant dies in response to the mating pheromone even in Ca$^{2+}$-containing media because of a deficiency of Ca$^{2+}$ influx and is rescued when high concentrations of CaCl$_2$ are supplemented. Other yeast mutants that show the mid phenotype have been identified. Those include mutants deficient in calmodulin (18), Ca$^{2+}$/calmodulin-dependent protein kinase (18), calcineurin (18, 19), and a homologue of the α$_1$ subunit of mammalian, voltage-gated Ca$^{2+}$ channels (20, 21).

The *MID1* gene encodes a stretch-activated Ca$^{2+}$-permeable channel composed of 548 amino acid residues and has four hydrophobic segments named H1–H4 and 16 putative N-linked glycosylation sites (17). Although the Mid1 polypeptide has no hydrophobic similarity to known ion channels, the H2 and H4 segments are partially similar to the transmembrane seg-

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plasmids were rescued from Leu mants were plated onto SD-leucine plates, and then the gap-repaired mid1 seven alleles, the gap repair method (25) was employed. The plasmid prepared by the method of Sambrook by adding 100 μM sodium pantothenate. SD.Ca100 medium was prepared – deficient medium SD-Ca, CaCl2 was omitted, and calcium pantothenate – was replaced by sodium pantothenate. SD.Ca100 medium was prepared by adding 100 μM CaCl2 to SD-Ca medium. LB and 2× YT media were prepared by the method of Sambrook et al. (24).

Isolation of mid1 Alleles by the Gap Repair Method—To isolate mid1 alleles, the gap repair method (25) was employed. The plasmid YCPMID1–21 (17) containing the wild-type MID1 gene and the selection marker LEU2 was digested with restriction enzymes, HindIII and SnaBI, and digested plasmids without MID1 were introduced into seven mid1 mutants by a lithium acetate method (26). The transformants were plated onto SD-leucine plates, and then the gap-repaired plasmids were rescued from Leu+ transformants by the method of Holm et al. (27). The recovered plasmids were propagated in Escherichia coli, purified, and subjected to DNA sequencing (28) with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and a DNA sequencer, DSQ 2000L (Shimadzu, Kyoto, Japan).

In Vitro Random Mutagenesis with Hydroxylamine—The YCPMID1–23 plasmid (17) containing the wild-type MID1 gene was mutagenized with hydroxylamine (HA) with a mutation frequency of 0.9% by the method of Adams et al. (29). The HA solution contained 0.35 g of HA.HCl and 0.09 g of NaOH in 5 ml of H2O. Ten micrograms of YCPMID1–23 was added to 500 μl of the HA solution sterilized through a filter (Millex-GV, 0.22 μm, Millipore Corp., Bedford, MA), and the mixture was incubated for 20 h at 30 °C. On the following day, 10 μl of 5 M NaCl and 50 μl of 1 mg/ml bovine serum albumin were added to the mixture to stop the reaction, and the plasmid DNA was purified by ethanol precipitation. The mutated plasmids were digested with HindIII and SnaBI, and purified plasmids from each single colony were introduced into the E. coli strain XL1-Blue for amplification. The amplified plasmids were introduced into the mid1-1 mutant, and the viability of the transformants was examined by the methylene blue plate method (see below). The plasmids incapable of complementing of the mid1-1 mutant were treated with BamHI and Xhol to cut out the DNA fragment containing the MID1 coding region as well as the 5' and 3'-flanking regions (150 bp upstream of the initiation codon and 858 bp downstream of the termination codon). The DNA fragments were inserted into the vector portion of unmutagenized YCPMID1–23 treated with BamHI and Xhol and tested again for complementing activity. This procedure eliminates possible mutations in the vector. The plasmids finally identified were subjected to DNA sequence analysis (28).

Construction of mid1 Mutants by Site-directed Mutagenesis—Site-directed mutagenesis of the MID1 gene in the plasmid YCPMID1–23 (17) was performed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the method described by the manufacturer. Mutagenic primers (Table II) were synthesized to order by Sawady Technology Co., Ltd. (Tokyo, Japan).

Determination of mid1 Complementation Activity by Methylene Blue Methods—For screening mutants bearing a nonfunctional mid1 gene after in vitro random mutagenesis with HA, we employed a methylene blue plate (MBP) method (17). This method is based on the fact that viable cells, but not inviable cells, reduce the colorless methyl blue to the blue colorless leucomethyl blue (30, 31). Thus, the viable cells remain white, and inviable cells stain blue. Cells of the mid1 mutant transformed by HA-treated YCPMID1–23 (LEU2) were plated onto SD plates lacking leucine to give about 1000 colonies/plate and incubated for 2–3 days at 30 °C. The Leu+ transformants on the plates received 0.1 ml of 10 m NaCl and 7 ml of SD medium containing 0.5% agar and 0.01% methylene blue and were incubated for several days. At this stage, the colonies mainly with dead cells become blue but still have viable cells in parts, whereas colonies formed by cells transformed with intact YCPMID1–23 remain white. The blue colonies were selected, suspended in 0.1 ml of SD medium, streaked onto an SD plate, and incubated for 2 days. To avoid selecting cells contaminated from other colonies at the step of preparing 0.5% agar described above, six colonies from each single blue colony were then subjected to quantitative viability assay using the methylene blue liquid (MBL) method (14).

In the MBL method, 0.1 ml of exponentially growing culture in SD.Ca100 medium or that treated with a-factor for 4 or 8 h was mixed with an equal volume of 0.01% methylene blue, 2% sodium citrate solution, and the number of viable white cells and inviable blue cells was counted under a differential interference-contrast microscope.

**Fig. 1. Schematic representation of the Mid1 protein.** A, the total length of the Mid1 protein composed of 548 amino acid residues with four hydrophilic segments, H1–H4. B, the carboxyl-terminal region that contains three possible functional motifs. Bars, the positions of the motifs; numerals, the positions of the first and last amino acid residues of each motif. Open circles, cysteine residues.
was measured according to the method described by Iida et al.

Preparation of Anti-Mid1 Antibodies and Immunoblot Analysis—
Rabbit polyclonal antibodies to the Mid1 protein were raised against the glutathione S-transferase-Mid1 (GST-Mid1) fusion protein. To obtain the GST-Mid1 protein, we constructed pGEX-6P-2-MID1, an E. coli plasmid that expresses it under the control of the tac promoter. Fusion of Mid1 with GST was necessary, because Mid1 was toxic to the cells when expressed alone. pGEX-6P-2 (Amersham Biosciences) was digested with BamHI and ligated with a linker DNA containing the NsiI site (5'-pGATCCATCATCTGCAGATGCATG-3', where the NsiI sites are underlined) to create the pGEX-6P-2-N vector. pGEX-6P-2-MID1 was constructed by inserting the 1.7-kb NsiI-NsiI fragment that contains the entire MID1 open reading frame from pBSMID1-N2 into the NsiI site of pGEX-6P-2-N. The E. coli strain JM109 was transformed with pGEX-6P-2-MID1, and the transformant was incubated to the stationary phase in 2x YT medium at 37°C. The cells were harvested and disrupted by sonication. The protein extracts were fractionated by centrifuging at 20,000 × g, and the pellets were washed twice with PBS containing 2% Triton X-100 (Bio-Rad). Because the GST-Mid1 fusion protein was present in the inclusion body, 7M urea solution made in 50 mM Tris-HCl (pH 8.5), 10 mM dithiothreitol, and a 1% SDS sample buffer containing synthetic medium without histidine or adenine. The transformants were streaked onto agar plates containing synthetic medium without histidine or adenine. The transformants were also examined for β-galactosidase activity by the filter method (36) and the quantitative liquid method (37).

RESULTS

Analysis of mid1 Alleles Induced by EMS in Vivo Reveals the Importance of the H2 and Carboxyl-terminal Domains—To identify the amino acids required for the function of Mid1, we isolated and analyzed the seven EMS-induced mid1 alleles that we reported previously (17). The mid1 alleles were recovered by the gap repair method as described under “Experimental Procedures,” and the gap-repaired mid1 alleles that did not complement the mid phenotype (see above) of the corresponding mid1 mutants were selected and then subjected to DNA sequencing and computational polypeptide analysis. As shown in Fig. 2, the mid1-1 allele product had two mutations, Y254H (TAC to CAC) and W441Op (TGG to TGA). Analysis of the single mutations generated by site-directed mutagenesis showed that the Y254H mutation was a silent mutation and that the W441Op mutation was responsible for the mid phenotype associated with the mid1-1 mutation (data not shown). The mid1-2 and mid1-5 allele products had a common mutation, Y254H (TAC to CAC), indicating that the two alleles have no loss-of-function mutation in the coding region. The mid1-3 allele product had G104D (GCC to GAC) and Y254H (TAC to CAC) mutations. Again, analysis of the single mutations generated by site-directed mutagenesis showed that only the G104D mutation was responsible for the mid phenotype associated with the mid1-3 mutation (data not shown). It is of interest that Gly104 is located in the hydrophobic segment H2. The mid1-4 allele product had Y254H (TAC to CAC) and W441Am (TGG to TAG) mutations. Note that this allele product is the same as the mid1-1 allele product with different stop codons. The mid1-6 allele had a deletion of a nucleotide in the codon for Met109, resulting in a frameshift mutation producing a protein lacking about four-fifths of the Mid1 polypeptide after Gly108 with two extra amino acid residues, Cys and Pro. The mid1-7 allele product had the C498Y (TGC to TAC) mutation only. These results indicate that Gly104, Cys498, and the carboxyl-terminal region (Trp 441 to the Gal4 DNA-binding domain; pGAD-Mid1 H2–H4 carrying the H2–H4 region fused to the Gal4 activation domain; and a positive control plasmid, pYM870, carrying the full-length Gal4 (35)).

Combinations of control and fusion plasmids were transformed into the PJ69-4A strain (34). Transformants were streaked onto agar plates containing synthetic medium without histidine or adenine. The transformants were also examined by β-galactosidase activity by the filter method (36) and the quantitative liquid method (37).

Essential Carboxyl-terminal Regions of the Mid1 Channel

TABLE I

Yeast strains used in this study

| Strain       | Genotype        | Ref. |
|--------------|-----------------|------|
| H301-1       | MATa mid1-1 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-2       | MATa mid1-2 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-3       | MATa mid1-3 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-4       | MATa mid1-4 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-5       | MATa mid1-5 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-6       | MATa mid1-6 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |
| H301-7       | MATa mid1-7 his3Δ1 leu2-3,112 trp1-289 ura3-52 set1-2 | 17   |

(Yamashita, Tokyo, Japan). The viability was expressed as the number of viable white cells as a percentage of the total number of cells. Colonies that produced about 30% viable culture 8 h after receiving α-factor were selected for further analyses.

Analysis of mid1 Alleles Induced by Hydroxylamine in Vitro Reveals the Importance of the Carboxyl-terminal Region—To further identify the amino acids or regions required for the function of Mid1, we analyzed hydroxylamine-induced mid1 alleles. The plasmid YCPMID1–23 (17) was mutagenized with hydroxylamine in vitro with a mutation frequency of 0.9% according to Adams et al. (29), and the mutagenized plasmid was examined for the ability to complement the mid1-1 mutation by the MBL method followed by the MBL method as described under “Experimental Procedures.” Thirty-seven plas-
mids (designated HA1–HA37) that could not complement the mid1-1 mutation were isolated and subjected to DNA sequencing, and their mutation sites were investigated. As shown in Table III, 31 plasmids had the same base substitution (i.e. G to A) at a substitution at nucleotide 1323, generating the W441Op (TGG to TGA) mutation. It is notable that this mutation results in the carboxyl-terminal truncation of Mid1 as the mid1-1 and mid1-4 mutations. HA19 had deletion of G at nucleotide 607, causing a frameshift mutation after Leu202.

HA20 had the Y457Am (TAC to TAG) mutation. HA23 had two mutations, P110S (CCC to TCC) and W211Op (TGG to TGA). Because the above results indicate the putative portion of Mid1—essential carboxyl-terminal regions of the Mid1 channel—and their mutation sites were investigated. As shown in Table III, 31 plasmids had the same base substitution (i.e. G to A) at a substitution at nucleotide 1323, generating the W441Op (TGG to TGA) mutation. It is notable that this mutation results in the carboxyl-terminal truncation of Mid1 as the mid1-1 and mid1-4 mutations. HA19 had deletion of G at nucleotide 607, causing a frameshift mutation after Leu202.

Progressive Carboxyl-terminal Truncation Defines an Essential Portion of Mid1—Because the above results indicate the importance of the carboxyl-terminal region for the function of
To examine the possible contribution of the putative casein kinase 2 phosphorylation site to Mid1 function, we further carried out mutational analysis. The results showed that the mutant alleles, in which the putative phosphorylation site Thr\textsuperscript{511} was substituted with Ala or Val (T511A and T511V), complemented the mid phenotype of the mid1-1 mutant (Fig. 5A), indicating that phosphorylation of this site is not a factor for Mid1 function.

We then examined the possible involvement of the sheet-turn-sheet structure in Mid1 function by replacing Tyr\textsuperscript{520} with Ala or Gly to disrupt this structure. As shown in Fig. 5A, both the Y520A and Y520G mutant alleles did not complement the mid1-1 mutation, suggesting that this structure may be required for Mid1 function. In contrast, carboxyl-terminal truncation into this structure up to Phe\textsuperscript{522} did not affect the complementing ability of the truncated alleles (Fig. 5B).

Finally, this carboxyl-terminal truncation experiment defined the carboxyl-terminal boundary for the shortest functional Mid1. The F522Am allele, but not the N521Am allele, complemented the mid phenotype (Fig. 5B), indicating that the site between Asn\textsuperscript{521} and Phe\textsuperscript{522} is the boundary. In other words, the Met\textsuperscript{1}-Asn\textsuperscript{521} polypeptide is the shortest functional Mid1 in terms of carboxyl-terminal truncation.

**Complementing Ability Correlates with Ca\textsuperscript{2+} Accumulation Activity**—Because another phenotype of the mid1 mutants has a low activity in Ca\textsuperscript{2+} uptake (17), we measured the Ca\textsuperscript{2+} accumulation in cells having mid1 mutations at or near the boundary as described above (i.e., at or near Asn\textsuperscript{521} and Phe\textsuperscript{522}). Fig. 6 shows that L510Am, Y520A, Y520G, Y520Am, and N521Am, which do not complement the mid1 mutation, had low activities in accumulating Ca\textsuperscript{2+}, comparable with or close to a mid1-1 mutant level, while F522Am and T526Op, which complement the mid phenotype, accumulated Ca\textsuperscript{2+} normally. This result indicates that the complementing ability of the mid phenotype correlates with the activity of Ca\textsuperscript{2+} accumulation.

**Seven Cysteine Residues Are Required for Mid1 Function**—The C498Y mutation found in the mid1-7 allele (Fig. 2) implies that some cysteine residues in the carboxyl-terminal region are important. We therefore exchanged each one of the cysteine residues in that region for alanine residues and examined the ability of each mutant allele to complement the mid1-1 mutation. As shown in Fig. 7, C417A, C431A, C434A, and C498A mutants did not have the complementing ability just like a negative control (the mid1-1 mutant itself), whereas C443A, C450A, and C487A had full complementing ability. C491A, C450A, and C526A, which do not complement the mid1 mutation, had low activities in accumulating Ca\textsuperscript{2+}, comparable with or close to a mid1-1 mutant level, while F522Am and T526Op, which complement the mid phenotype, accumulated Ca\textsuperscript{2+} normally. This result indicates that the complementing ability of the mid phenotype correlates with the activity of Ca\textsuperscript{2+} accumulation.

**Discussion**

We have identified the carboxyl-terminal region and amino acid residues important for the function of the Mid1 channel by conventional mutagenesis with EMS, in vitro random mutagenesis with hydroxylamine, and site-directed in vitro mutagenesis. A previous study has indicated that the carboxyl-terminal region after the H4 domain is hydrophilic, cysteine-rich, and intracellular (17). Thus, the region has been postulated to be a regulatory region for this channel. Our present study has clearly shown that this region is essential for Mid1 function. This region is indeed required for the complementing ability and Ca\textsuperscript{2+} uptake activity, and the essential carboxyl-terminal region excludes the distal portion from Phe\textsuperscript{522} to Thr\textsuperscript{541}.

The essential carboxyl-terminal region has at least three motifs, including a putative casein kinase 2 phosphorylation motif from Thr\textsuperscript{511} to Asp\textsuperscript{514}, an EF-hand-like structure from...
Phe408 to Val445, and a sheet-turn-sheet motif from Thr 512 to Thr526.

As mentioned before, the putative casein kinase 2 phosphorylation motif is not required for Mid1 function (Fig. 5A). We have found by the 45Ca2+/H11001 overlay technique that the EF-hand-like structure (Phe408–Val445) does not bind 45Ca2+/H11001 under the conditions under which calmodulin, a positive control, binds it (data not shown), suggesting that this structure is not a high affinity Ca2+/H11001-binding site. This is probably because the structure has crucial amino acid deletions and substitutions. However, this structure could still be important, because Cys417, Cys431, and Cys434 in the E- or F-like helix structure are essential for Mid1 function (Fig. 7). In addition, it is known that the distorted EF-hand can function as a protein-protein interaction site (38–40). Thus, the EF-hand-like structure of the Mid1 channel could contribute to interact with other proteins or with Mid1 protein itself.

To examine the possible self-interaction between the Mid1 proteins via the carboxyl-terminal regions, we performed the Gal4-based two-hybrid assay on the following three combinations (i.e. interactions between (a) the carboxyl termini, (b) the carboxyl terminus and the central domain (H2–H4), and (c) the H2–H4 domains (for details, see “Experimental Procedures”). The results were all negative (data not shown). Therefore, we still do not know unambiguously if the carboxyl-terminal region as well as the central region between H2 and H4 contributes to a possible self-interaction.

This study has revealed the importance of cysteine residues in the essential carboxyl-terminal region. There are 10 cysteine residues in this region, and they are all conserved in the Mid1 homologue of a fission yeast Schizosaccharomyces pombe.

### Table III

| Base change | Amino acid change | Type of mutation | Plasmid |
|-------------|------------------|-----------------|---------|
| G-A         | W441stop (Op)    | Nonsense mutation | HA1–HA5, HA8, HA9, HA11–HA18, HA21, HA22, HA24–HA37 |
| G-A         | W211stop (Op)    | Nonsense mutation | HA23    |
| C-T         | P110S            | Missense mutation | HA23    |
| C-G         | Y457stop (Am)    | Nonsense mutation | HA20    |
| G-lack      |                  | Frameshift mutation | HA19    |

### Fig. 3. Complementing ability of carboxyl-terminally truncated Mid1 proteins.

Cells expressing each one of the carboxyl-terminally truncated Mid1 proteins were incubated with 6 μM α-factor for 8 h, and the viability of the cells was examined by the MBL method. Am and Op indicate the nonsense mutations, amber and opal, respectively.

### Fig. 4. Expression of various mutant Mid1 proteins.

Cells harboring plasmids encoding wild type and various mutant Mid1 proteins were cultured to midlog phase and homogenized. A portion of each homogenate (10 μg of protein) was subjected to 7.5% SDS-PAGE followed by immunoblot analysis with antibodies to the Mid1 protein. Enolase, detected by rabbit polyclonal antibodies to yeast enolase (46), was used as a loading standard. A, a series of progressive carboxyl-terminal truncations; B, a series of alanine-scanning mutagenesis on the cysteine-rich regions.

### Fig. 5. Complementing ability of Mid1 proteins having a mutation in putative functional motifs.

A, cells expressing a mutant Mid1 protein having a mutation in a putative casein kinase 2 phosphorylation site (T511A and T511V) or that in a putative sheet-turn-sheet structure (Y520A and Y520G) were incubated with 6 μM α-factor for 8 h, and the viability of the cells was examined by the MBL method. Note that Tyr520 is in the turn structure. B, mutant Mid1 proteins that have a nonsense mutation in the putative sheet-turn-sheet structure were expressed in the mid1-1 mutant, and the viability of the cells was examined as described above.

Cys431, and Cys434 in the E- or F-like helix structure are essential for Mid1 function (Fig. 7). In addition, it is known that the distorted EF-hand can function as a protein-protein interaction site (38–40). Thus, the EF-hand-like structure of the Mid1 channel could contribute to interact with other proteins or with Mid1 protein itself.

To examine the possible self-interaction between the Mid1 proteins via the carboxyl-terminal regions, we performed the Gal4-based two-hybrid assay on the following three combinations (i.e. interactions between (a) the carboxyl termini, (b) the carboxyl terminus and the central domain (H2–H4), and (c) the H2–H4 domains (for details, see “Experimental Procedures”). The results were all negative (data not shown). Therefore, we still do not know unambiguously if the carboxyl-terminal region as well as the central region between H2 and H4 contributes to a possible self-interaction.

This study has revealed the importance of cysteine residues in the essential carboxyl-terminal region. There are 10 cysteine residues in this region, and they are all conserved in the Mid1 homologue of a fission yeast Schizosaccharomyces pombe pro-
This suggests that Cys 491 and Cys 531 take part in a common Cys 450, and Cys 487) are nonessential, and the other three and 2 h after the incubation, samples (200 μl) were filtered through Millipore filters (type HA; 0.45 μm) that had been presoaked in 5 mM CaCl2 and washed five times with the same solution. The radioactivity retained on the filters was counted. The radioactivity of cells incubated for 0 h was subtracted from that of cells incubated for 2 h.

FIG. 6. Ca2+ accumulation in cells expressing various mutant Mid1 proteins. Cells in the exponentially growing phase in SD-Ca100 medium were incubated with 185 kBq/ml 45CaCl2 (1.8 kBq/nmol). At 0 and 2 h after the incubation, samples (200 μl) were filtered through Millipore filters (type HA; 0.45 μm) that had been presoaked in 5 mM CaCl2 and washed five times with the same solution. The radioactivity retained on the filters was counted. The radioactivity of cells incubated for 0 h was subtracted from that of cells incubated for 2 h.

FIG. 7. Complementing ability of Mid1 proteins having a mutation in cysteine residues. Ten cysteine residues were substituted with alanine residues. The viability of the cells expressing a mutant Mid1 protein was determined as described in the legend to Fig. 3.

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