Molecular Dissection of the Hydrophobic Segments H3 and H4 of the Yeast Ca\(^{2+}\) Channel Component Mid1*§

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The Saccharomyces cerevisiae MID1 gene product, Mid1, is composed of 548 amino acid residues, has four relatively hydrophobic segments named H1–H4, and functions as a Ca\(^{2+}\)-permeable, stretch-activated channel when expressed in mammalian cells. In some conditions Mid1 cooperates with Cch1, a yeast homolog of the α1 subunit of mammalian voltage-gated channels. To identify the important regions or amino acid residues necessary for Mid1 function, we employed in vitro site-directed mutagenesis on H3 and H4 of Mid1 and expressed the resulting mutant genes in a mid1 null mutant to examine whether the mutant gene products are functional or not in vivo. Mutant Mid1 proteins lacking the whole H3 or H4 segment, H3De or H4De, did not complement the lethality and low Ca\(^{2+}\) accumulation activity of the mid1 mutant, although their localization and contents appeared to be normal, indicating that H3 and H4 are required for Mid1 function itself. Single amino acid exchange experiments on individual amino acid residues of H3 and H4 showed that 10 of 20 residues in H3 and 14 of 23 residues in H4 were important for the normal function of Mid1. In particular, we found four severe loss-of-function mutations, D341E, F356S, C373D, and C373R, and two interesting mutations leading to a high level of Ca\(^{2+}\) accumulation with a slightly low complementing activity, G342A and Y355A. The importance of these amino acid residues will be discussed.

The MID1 gene product, Mid1, is an N-glycosylated, integral membrane protein required for viability of differentiated yeast cells and Ca\(^{2+}\) influx induced by the mating pheromone α-factor (1). Cells lacking the MID1 gene die because of limited Ca\(^{2+}\) influx when incubated with α-factor. This phenotype is called the mid1 phenotype for the mating pheromone-induced death phenotype. Electrophysiological and cell biological studies on Chinese hamster ovary cells, Balb/c 3T3 cells, and COS-7 cells expressing Mid1 have revealed that it functions as a Ca\(^{2+}\)-permeable stretch-activated channel (2, 3). The mating pheromone leads its target cells to differentiate into cells (so-called “shmoo”) having a mating projection at which the plasma membrane and the cell wall are degraded and regenerated (4, 5). Concomitantly with the formation of the mating projection, Mid1-dependent Ca\(^{2+}\) influx is stimulated (1, 6, 7). Thus, Mid1 might function in sensing membrane stretch and generating Ca\(^{2+}\) signals during the mating process. Although stretch-activated channels or mechanosensitive channels are known to play a critical role in touch sensation, hearing, balance, detecting gravity, and sensing osmotic changes, little is known about their molecular structures and biochemical properties.

Genetic and cell biological studies have revealed that Mid1 coimmunoprecipitates and works with Cch1, a yeast homolog of the pore-forming α1 subunit of mammalian voltage-gated Ca\(^{2+}\)-channels (8–10). The voltage-gated Ca\(^{2+}\) channels are heteromultimeric proteins consisting of α1, cytoplasmic β, and non-pore-forming transmembrane α2δ and γ subunits (11). These non-pore-forming subunits dramatically influence the properties and surface expression of the channels (12–16). Although the channel activity of Cch1 has not yet been revealed experimentally and Mid1 has no homology to the auxiliary subunits, Mid1 has been shown to cooperate with Cch1 in mating pheromone-induced uptake (8, 9, 17), store-operated or capacitative Ca\(^{2+}\) entry (10), endoplasmic reticulum stress-induced Ca\(^{2+}\) uptake (18), and a hyperosmotic stress-induced increase in cytosolic Ca\(^{2+}\) (19). On the other hand, it has been shown recently that Mid1, but not Cch1, is required for an antiarrhythmic drug amidarone-induced increase in cytosolic Ca\(^{2+}\) mainly caused by Ca\(^{2+}\) influx (20). Mid1 is also involved in a hexose-induced, transient elevation of cytosolic Ca\(^{2+}\) (21). Therefore, Mid1 might function as an stretch-activated channel alone in some cellular situations and as a regulator of another channel composed of Cch1 in other cellular situations. This speculation remains to be proven at the molecular level.

Although the physiological roles of Mid1 have been elucidated as described above, the structure-function relationship of this protein remains unclear. The Mid1 polypeptide is composed of 548 amino acid residues and could form a homotetramer (see supplemental material in Ref. 2), having four hydrophobic segments, H1–H4 (1) (see Fig. 1). It is uncertain whether these segments are transmembrane domains. H1 is probably a signal peptide (1). Computational analysis with the TMPred program (available at www.ch.embnet.org/software/TMPRED_form.html) suggests that H3 and H4 are possible transmembrane helices. The hydrophobic profile of H3 is similar to that of the pore-forming regions of several cation channels (see supplemental material in Ref. 2). H4 is partially homologous to the S3/H3 membrane-spanning domain of several ion channels (1). Protease protection experiments on intact cells have revealed that Mid1 is present in the plasma membrane and that its C-terminal region is in the cytoplasm (1). The C-terminal, cytoplasmic region downstream from H4, including the cysteine-rich regions, is essential for Mid1 function (22).
In this study, to explore the structure-function relationship of H3 and H4, we mutated each one of all 20 and 23 amino acid residues in H3 and H4, respectively, and tested the effects of these single point mutants on Mid1 function by examining the ability of the mutant proteins to complement the mid1 phenotype and low Ca\(^{2+}\) accumulation of the mid1 mutant. From these analyses, we identified several key amino acid residues and their clusters that potentially contribute to Ca\(^{2+}\) permeability.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The yeast strains used in this study are listed in Table I. Rich media and a synthetic medium, SD, were prepared as described previously (23). Yeast nitrogen base was prepared according to the formula given in the Difco manual (24). Because SD medium contains 680.2 μM CaCl\(_2\) and 0.8 μM calcium pantothenate, in the Ca\(^{2+}\)-deficient medium SD–Ca, CaCl\(_2\) was omitted, and calcium pantothenate was replaced with sodium pantothenate. SD Ca100 medium was prepared by adding 100 μM CaCl\(_2\) to SD–Ca medium. Synthetic sporulation medium contained 6.7 g of yeast nitrogen base and 10 g of potassium acetate/sulfite/mediator. These media were supplemented with the appropriate nutrients as described previously (23). The sporulation medium contained 10 g of potassium acetate/sulfite/mediator.

Arabidopsis thaliana strain used was XL1-Blue (Stratagene). Competent cells were prepared according to the methods described previously (25). Luria-Bertani medium and terrific broth were prepared as described previously (26). When required, ampicillin was added at 50 μg ml\(^{-1}\).

Site-directed Mutagenesis—To construct plasmids with the MID1 gene having deletion mutations or single mutations, site-directed mutagenesis was employed. The mutagenic primers were ordered from Invitrogen, and their DNA sequences are listed in Table II (supplemental data available at JBC on-line). PCR was performed with Pfu polymerase (Stratagene) and YCpMID1-23 or YEpMID1-GFP1 (Table I) as a PCR template. The PCR Thermal Cycler MP (Takara) was used for PCR. The low copy plasmid YCpMID1-23 and its derivatives were used for measuring the viability of cells and Ca\(^{2+}\) accumulation and immunoblot analysis; the multicopy plasmid YEpMID1-GFP and its derivatives were used for detecting Mid1-GFP by confocal microscopy and immunoblot analysis. Successful mutagenesis was confirmed by DNA sequencing using an ABI Prism Automated Sequencing Kit and an ABI immunoblot analysis. Successful mutagenesis was confirmed by DNA sequencing, and tetrad analysis. Immunoblot analysis confirmed further that the Mid1 protein was not produced from the mid1-Δ5 allele at all.

Transformation of Saccharomyces cerevisiae Cells—The S. cerevisiae strain H311 was transformed by various plasmids whose selection marker was LEU2 (Table I) according to the method described previously (27), with minor modifications. The transformants were selected on agar plates containing SD medium supplemented with 20 μg/ml uracil and 20 μg/ml tryptophan at 30 °C for 3 days.

Activity of the Mutant Mid1 Proteins—To examine the activity of the mutant Mid1 proteins, the viability and Ca\(^{2+}\) accumulation of cells producing the proteins were measured by the methods described previously (1). The mating pheromone α-factor was ordered from the Center for Analytical Instruments, National Institute for Basic Biology, Okazaki, Japan.

GFP Fluorescence Imaging—The Mid1-GFP fusion proteins having mutations were expressed in the strain H311. Cells in the exponential phase in SD medium were harvested and placed on slide glasses covered with 1% poly-l-lysine hydrobromide. Subsequently, the slide glasses were sealed under a coverslip with nail polish. GFP images were observed using a confocal fluorescence microscope equipped with Nikon TE 300 (Nikon) in conjunction with Micro Radiance (Bio-Rad). We used Nikon Plan apo 60x1.40 Oil (Nikon) as an objective lens. Images were processed using Adobe Photoshop 5.0 (Adobe Systems).

Preparation of Cell Extracts and Immunoblot Analysis—The methods of cell extract preparation were described previously (1) and used with slight modifications. An improved immunoblotting technique was employed (28). Cell extracts containing the Mid1 protein were applied to a 7.5% SDS-polyacrylamide gel and detected using rabbit polyclonal antibodies against the glutathione S-transferase-Mid1 (GST-Mid1) fusion protein at a 1:2,000 dilution (22). Enolase was detected using rabbit polyclonal antibodies against yeast enolase at a 1:5,000 dilution (29) and used for an internal marker for the amount of protein applied.

Statistical Analysis—Statistical significance was determined using an unpaired Student’s t test, with a maximum p value of <0.05 required for significance.

RESULTS

H3 Is Essential for Mid1 Function—To determine whether H3 is essential for Mid1 function, a mutant Mid1 protein lacking the whole H3 segment from Ile\(^{387}\) to Phe\(^{396}\), H3De, was constructed by site-directed mutagenesis using the low copy plasmid YCpMID1-23 (1) as a template and expressed in the mid1-Δ5 mutant. The mutant expressing H3De, the H3De mutant, was incubated with α-factor for 8 h and examined for the viability of the cells. The result shows that the H3De mutant protein did not complement the mid1 phenotype of the mid1-Δ5 mutant (Fig. 2A). Because the mid1 mutations result in a low activity in mating pheromone-induced Ca\(^{2+}\) accumulation (1), we measured Ca\(^{2+}\) accumulation in the H3De mutant exposed to α-factor for 2 h. Ca\(^{2+}\) accumulation in the H3De mutant was low, like that in the mid1-Δ5 mutant containing the vector pRS315 (Fig. 2B). The result suggests that the H3 segment is required for Mid1 function.

To identify important amino acid residues in H3, all 20 amino acid residues in this segment were mutated individually by site-directed mutagenesis with the following rules. All of the nonpolar amino acids were replaced by a polar amino acid, Ser, and the polar amino acids were replaced by a small, nonpolar

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1 The abbreviations used are: GFP, green fluorescence protein; GST, glutathione S-transferase.
amino acid, Ala. Gly is classified as a polar amino acid and was thus replaced by Ala. Because it has been reported that the negatively charged amino acids Glu and Asp in the transmembrane domain contribute to ion selectivity in voltage-gated Ca\textsuperscript{2+}/H\textsuperscript{+} channels (30, 31) and ECaC (32, 33) and to interaction with channel blockers (34, 35), the remarkable Asp341 was replaced by each of 7 amino acids, Glu, Lys, Arg, Thr, Asn, Ala, and Leu, and the positively charged amino acid His353 was also replaced by each of 3 amino acids, Asp, Arg, and Ala. The mutant genes bearing these single amino acid substitutions were then introduced into cells of the mid1\textsuperscript{-}/H\textsuperscript{+} mutant, and the resulting transformants were examined for viability and Ca\textsuperscript{2+} accumulation after exposure to a-factor. The results showed that the most notable Mid1 mutant protein was D341E, which had almost completely lost both the activity of complementing the mid phenotype and Ca\textsuperscript{2+} accumulation activity (Fig. 2). F356S also lost the complementing activity completely (Fig. 2A) but still had Ca\textsuperscript{2+} accumulation activity near the wild-type level (Fig. 2B). G342A, I349S, V354S, and Y355A had slightly lower complementing activities than the wild-type protein, but two of which, G342A and Y355A, had greater Ca\textsuperscript{2+} accumulation activity than the wild-type protein, whereas the remainder

| Strains or plasmids | Relevant markers | Reference |
|---------------------|-----------------|-----------|
| S. cerevisiae strains | MATa his3\textsuperscript{-}\Delta1 leu2-3,112 trp1-289 ura3-52 sst1-2 | 1 |
| H207 | MATa his3\textsuperscript{-}\Delta1 leu2-3,112 trp1-289 ura3-52 sst1-2 | 1 |
| H311 | MATa his3\textsuperscript{-}\Delta1 leu2-3,112 trp1-289 ura3-52 sst1-2 mid1\textsuperscript{-}/H\textsuperscript{+}::HIS3 | This work |
| Plasmids | | |
| EpMID1-31-P-HIS3-2 | f\textsuperscript{1} amp\textsuperscript{1} mid1\textsuperscript{-}\Delta4::HIS3 | 1 |
| EpMID1-\Delta5 | f\textsuperscript{1} amp\textsuperscript{1} mid1\textsuperscript{-}\Delta5::HIS3 with a stop codon mutation at Ile\textsuperscript{2} | This work |
| YEpplac112 | 2 \mu\textsuperscript{m} amp\textsuperscript{1} TRP1 | This work |
| YEpplac112-MID1 | 2 \mu\textsuperscript{m} amp\textsuperscript{1} TRP1 MID1 | This work |
| YEpplac112-MID1-\Delta5 | 2 \mu\textsuperscript{m} amp\textsuperscript{1} TRP1 mid1\textsuperscript{-}\Delta5::HIS3 | This work |
| pH315 | f\textsuperscript{1} amp\textsuperscript{1} LEU2 | 45 |
| YEpMID1-23 | f\textsuperscript{1} amp\textsuperscript{1} LEU2 MID1 | Gift from Chikako Miyawaki\textsuperscript{a} |
| YEpMID1-GFP | 2 \mu\textsuperscript{m} amp\textsuperscript{1} TRP1 GFP | Gift from Chikako Miyawaki\textsuperscript{a} |
| YEpMID1-GFP-2 | Gift from Chikako Miyawaki\textsuperscript{a} |

\textsuperscript{a} C. Miyawaki, H. Iida, H. Tatsumi, and M. Sokabe, manuscript in preparation.

Fig. 2. Viability and Ca\textsuperscript{2+} accumulation of cells expressing a Mid1 protein having a mutation in H3. A, the viability of cells was determined by the methylene blue liquid method (1). Exponentially growing cells of various strains grown in SD.Ca100 medium were incubated with 6 \mu\text{g} \alpha\text{-factor for 8 h.} B, Ca\textsuperscript{2+} accumulation activity of cells. Exponentially growing cells of various strains grown in SD.Ca100 medium were incubated with 6 \mu\text{g} \alpha\text{-factor and 185 kBq/ml (1.8 kBq/nmol)} \textsuperscript{45}\text{CaCl}_2 \text{for 2 h. The shading represents the level of viability and Ca}^\text{2+} \text{accumulation of the cells expressing a mutant protein: black bars, significantly lower than wild-type; white bars, significantly higher; gray bars, essentially identical. The means \pm S.D. from at least three independent experiments performed in triplicate are given.} \text{p} < 0.05 \text{(significantly different from the wild-type cells value; unpaired Student's t test).}
had normal Ca\(^{2+}\) accumulation activity. In contrast, I337S, N339A, and D341A had slightly greater complementing activity than the wild-type protein, among which I337S had a significantly increased Ca\(^{2+}\) accumulation activity and the remainder had normal Ca\(^{2+}\) accumulation activity. The above observation that G342A and Y355A had a significantly lower complementing activity than the wild-type protein despite having a greater Ca\(^{2+}\) accumulation activity suggests at least two possibilities. One is that overaccumulation of Ca\(^{2+}\) may lower the viability of cells. The other is that Mid1, as a regulator, might activate Cch1 and inactivate another factor responsible for cell viability.

It is possible that some of the above mutations are dominant negative mutations rather than loss-of-function mutations. To test this possibility, the low copy plasmid containing severe mutations, such as H3De, D341E, or F356S, was introduced into wild-type cells, and the resulting transformants were examined for viability 8 h after the addition of \(\alpha\)-factor. The results showed that the viabilities of these transformants were normal (data not shown), indicating that the mutations tested are recessive and loss-of-function ones.

H4 Is Essential for Mid1 Function—A mutant Mid1 protein lacking the whole H4 segment from Leu\(^{366}\) to Gly\(^{388}\), H4De, lost the activity of complementing the \(\text{mid}\) phenotype as well as Ca\(^{2+}\) accumulation activity as did the H3De protein under the same conditions (Fig. 3). To identify important amino acid residues in H4, all 23 amino acid residues in the segment were mutated individually by site-directed mutagenesis with the same rules as those in H3. Cys\(^{373}\) was replaced by each of 3 amino acids, Asp, Arg, and Ala, because Cys is a highly reactive amino acid residue. The results showed that the most marked mutant proteins were C373D and C373R (Fig. 3). The two proteins did not have either the complementing or Ca\(^{2+}\) accumulation activities at all. The 12 mutant proteins, F368S, L370S, D371E, F372S, D375E, A377S, Y378A, V380S, P381A, T382A, S383A, and G388A, had slightly lower complementing activity than the wild-type protein, although these proteins seemed to have essentially the same Ca\(^{2+}\) accumulation activity as the wild-type protein. In contrast, S384A had lower Ca\(^{2+}\) accumulation activity, although it had essentially the same complementing activity as the wild-type protein. The three severe mutations, H4De, C373D, and C373R, had no dominant negative effects on the viabilities of wild-type cells (data not shown), indicating that these mutations are loss-of-function ones.

H3 and H4 May Not Be Required for the Localization of the Mid1 Protein—The above results showing that the Mid1 proteins having a deletion of or a single mutation in H3 and H4 did not complement the \(\text{mid}\) phenotype suggest that these segments and amino acid residues are required for Mid1 function. However, it is also possible that those results are caused by mislocalization of the mutant proteins. To examine this possibility, the Mid1 protein fused with GFP at the C terminus, Mid1-GFP, was used for observing the protein localization by confocal fluorescence microscopy. The Mid1 protein is an integral membrane protein and present at the plasma membrane (1). It has also been shown that the Mid1 protein localizes at the endoplasmic reticulum (ER) membrane as well as the

![Figure 3: Viability and Ca\(^{2+}\) accumulation of cells expressing a Mid1 protein having a mutation in H4. The viability of cells (A) and Ca\(^{2+}\) accumulation activity of cells (B) are shown. Experimental conditions and the meanings of the bars are the same as those in Fig. 2. The means ± S.D. from at least three independent experiments performed in triplicate are given. *p < 0.05 (significantly different from the wild-type cells value; unpaired Student’s t test).](https://example.com/figure3)
plasma membrane, as revealed by indirect fluorescence microscopy. The wild-type Mid1-GFP produced from low- and multicity plasmids has been shown to completely complement the mid phenotype and to localize at the plasma membrane and endoplasmic reticulum membrane normally, indicating that GFP and the overexpression has no effect on the activity and localization of the Mid1 protein. However, it should be noted that fluorescence images of Mid1-GFP produced from the low copy plasmid were very faint and hard to be photographed. In addition, we have tried to examine the localization of the Mid1 protein produced from its gene on the intrinsic chromosome or on a low copy plasmid by indirect fluorescence microscopy using polyclonal anti-Mid1 antibodies, but we failed because of the very small expression levels of Mid1. We therefore examined the localization of Mid1-GFP produced from the multicopy plasmid thereafter.

By using the gene encoding the wild-type Mid1-GFP on the multicopy plasmid, the deletions or single mutations that had produced the mutant phenotypes shown in Figs. 2 and 3 were introduced by exactly the same methods as those used for the gene encoding the Mid1 proteins. Figs. 4A and 5A show that the wild-type Mid1-GFP localized on the plasma membrane and the endoplasmic reticulum membrane as expected, confirming the previous results, and Figs. 4B and 5B show that GFP itself was present in the cytoplasm. Fluorescence images for the localization of all the mutant Mid1-GFPs tested were essentially the same as those of the wild-type Mid1-GFP (Figs. 4 and 5), suggesting that the phenotypes of the mutant Mid1 proteins presented in Figs. 2 and 3 were not caused by the mislocalization of the mutant Mid1 proteins.

**DISCUSSION**

We have shown in this study that the H3 and H4 segments are necessary for Mid1 function. Deletion of either H3 or H4 resulted in a complete loss of function. In addition, we individually evaluated the amino acid residues in H3 and H4 required for the full activity of Mid1. Before discussing the importance of these amino acid residues, we will speculate about the structure of a putative Ca2+ channel composed of Mid1. Our previous work has shown that Mid1 might form a homotetramer to make a channel (see supplemental material in Ref. 2). In addition, Mid1 functions in an amiodarone-induced increase in cytosolic Ca2+, whereas Cch1 does not (20). On the other hand, it has been shown that the phenotypes of mid1 and cch1 single mutants and a mid1 cch1 double mutant are essentially the same (10, 17, 18), and immunoprecipitation experiments have shown that Mid1 coprecipitates with Cch1 (10), suggesting that Mid1 and Cch1 cooperate to work in some conditions. Two possible structure-function relationships could be hypothesized. One is that Mid1 might form a Ca2+ channel and serve a dual role as a stretch-activated channel mediating Ca2+ influx and as a stretch sensor, both of which might then generate a

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2 H. Yoshimura, S. Muto, and H. Iida, manuscript in preparation.
3 C. Miyawaki, H. Iida, H. Tatsumi, and M. Sokabe, manuscript in preparation.
Ca$^{2+}$ signal or a conformational change to activate a coupled Ca$^{2+}$ channel composed of Cch1. A mechanism analogous to this is well elucidated in the coupling between dihydropyridine receptors (the $\alpha_1$ subunits of voltage-gated Ca$^{2+}$ channels) and ryanodine receptors (sarcoplasmic reticulum Ca$^{2+}$ release channels) in vertebrate skeletal muscles (36, 37). The other hypothesis is that Mid1 might form no channel by itself and instead be a regulatory subunit protein of a Ca$^{2+}$ channel whose pore-forming subunit is Cch1. It is known that mammalian voltage-gated Ca$^{2+}$ channels have auxiliary subunits, $\alpha_2$, $\beta$, and $\gamma$, which regulate the activity of the pore-forming $\alpha_1$ subunit homologous to Cch1 (12–16). Because the structure of the putative Ca$^{2+}$ channel(s) composed of Mid1 and Cch1 is an open question at present, we would like to explain the data presented in this paper from the viewpoint of both hypotheses. In particular, four severe loss-of-function mutations, D341E, F356S, C373D, and C373R, and two mutations leading to a high level of Ca$^{2+}$ accumulation with a slightly low complementing activity, G342A and Y355A, are discussed below.

**Important Amino Acid Residues in H3**

Asp$^{341}$—When Asp$^{341}$ in H3 was replaced with Glu, Lys, Arg, Thr, Asn, Ala, or Leu, only the D341E mutation resulted in an almost complete loss of function, regarding maintenance of cell viability and Ca$^{2+}$ accumulation, whereas the other mutations did not deteriorate the function of Mid1 (Fig. 2). We examined three independent strains containing the D341E mutation and obtained the same results. In addition, we confirmed that the D341E mutant gene did not contain an additional mutation elsewhere in the MID1 gene. Therefore, these results suggest that the size of the residues is not important, but an appropriate strength of negative charge of this position may be. Glu has a greater negative charge than Asp ($pK_a$ 4.25 versus 3.86), and thus Ca$^{2+}$ would bind to Glu$^{341}$ too tightly to pass the channel, if Mid1 is the pore-forming subunit. It has been reported that Asp substitution in the EEEE locus, known as the filter of the L-type Ca$^{2+}$ channel, reduces ion selectivity by weakening ion binding affinity (38).
In terms of the second hypothesis that Mid1 is a regulatory protein of a Ca²⁺ channel containing Cch1, Asp⁵⁴⁴ could be an important amino acid residue that may interact directly or indirectly with Cch1 and activate it. Coexpression study of the human auxiliary subunit α₂β with the α₁ and β subunits has indicated that α₂β is required for an increase in the peak size of the N-type Ca²⁺ current (13). Interestingly, although Mid1 has no sequential similarity to the α₂β subunit at all, both Mid1 and α₂β are glycosylated plasma membrane proteins (1, 11, 39). Mid1 may directly up-regulate Cch1 like α₂β, and the loss-of-function mutation D341E may lead Mid1 not to activate Cch1.

**Gly²⁴²—**Cells of the G342A mutant have an interesting phenotype. They accumulate Ca²⁺ to a greater extent than wild-type cells and lose their viability (Fig. 2). One possibility is that the low viability is caused by much Ca²⁺ incorporated into this mutant. Because this mutation appeared not to affect the stability and localization of the G342A mutant protein (Figs. 4 and 6), the replacement of Gly²⁴² with Ala may be sufficient to cause a conformational change, either activating Mid1 itself or up-regulating Cch1 through the mutated Mid1. It has been suggested that a Gly residue and its adjacent amino acid residues in the gate play important roles in the gating mechanism in several ion channels. For example, the most conserved Gly²⁲ residue of *E. coli* MscL functions as the gate (40, 41). When Gly²⁴² is replaced with hydrophilic residues, the resulting mutant MscL channels become more mechanosensitive. On the other hand, a Gly residue in a transmembrane segment can be important amino acid residue that may interact directly or indirectly with MscL channels become more mechanosensitive. In this study, 13 of 23 amino acid residues in H4 were found to be conserved between 23 distinct mammalian Ca²⁺ channels regulated by non-pore-forming auxiliary transmembrane subunits, those studies would also shed light on the mechanism.

**H4 as a Possible Transmembrane Helix**

In this study, 13 of 23 amino acid residues in H4 were found to be required for the maintenance of viability and 2 for Ca²⁺ accumulation, but the degree of decreases in viability or Ca²⁺ accumulation was small for all of the mutants but Cys³⁷³ (Fig. 3). It is therefore unlikely that H4 contributes to the formation of the filter or the gate whose amino acid substitution is supposed to cause a large change in Ca²⁺ permeability. Thus, a further study is to examine whether H4 is an inner or outer helix.

If Mid1 is a regulatory subunit protein of Cch1 according to the second hypothesis, most amino acid residues in H4 would not be required for the interaction with and activation of Cch1. The C373D and C373R proteins completely lost Mid1 function regarding the viability and Ca²⁺ accumulation, but the C373A protein functioned quite normally. This indicates that Cys³⁷³ does not contribute to disulfide bonding (Fig. 3). Probably, the negative or positive charge of C373D and C373R interferes with the interaction between Mid1 and Cch1 or a protein regulating Cch1. It is also possible that the charges result in a serious conformational change leading to the inactivation of Mid1 not to activate Cch1.

In summary, using molecular genetic approaches, we have identified amino acid residues and clusters in the H3 and H4 segments important for Mid1 function. Especially, Asp⁵⁴¹, Gly²⁴² Tyr⁵⁴⁵, Phe³⁵⁶, and Cys³⁷³ have attracted a great deal of our attention. Further characterization of mutations in these amino acid residues should result in a better understanding of the molecular basis of the function of Mid1 as well as the structural and functional relationships between Mid1 and Cch1. In addition, because it remains to be elucidated how the pore-forming subunits of mammalian Ca²⁺ channels are regulated by non-pore-forming auxiliary transmembrane subunits, these studies would also shed light on the mechanism.

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