Molecular cloning in yeast by in vivo homologous recombination of the yeast putative α1 subunit of the voltage-gated calcium channel

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Abstract Saccharomyces cerevisiae has only one gene encoding a putative voltage-gated Ca\textsuperscript{2+} channel pore-forming subunit, CCH1, which is not possible to be cloned by conventional molecular cloning techniques using Escherichia coli. Here, we report the successful cloning of CCH1 in yeast by in vivo homologous recombination without using E. coli. Overexpression of the cloned CCH1 or MID1 alone, which encodes a putative stretch-activated Ca\textsuperscript{2+} channel component, does not increase Ca\textsuperscript{2+} uptake activity, but co-overexpression results in a 2- to 3-fold increase. Overexpression of CCH1 does not substantially complement the lethality and low Ca\textsuperscript{2+} uptake activity of a mid1 mutant and vice versa. These results indicate that co-overproduction of Cch1 and Mid1 is sufficient to increase Ca\textsuperscript{2+} uptake activity.

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1. Introduction

The Saccharomyces cerevisiae CCH1 gene product, Cch1, has sequence similarity to the α1 pore-forming subunit of the dihydropyridine-sensitive (L-type) family of mammalian voltage-gated Ca\textsuperscript{2+} channels [1,2]. In contrast, the MID1 gene product, Mid1, has no overall sequence similarity to known mammalian ion channels except for potential transmembrane segments [3], although it has stretch-activated Ca\textsuperscript{2+}-permeable channel activity when expressed in mammalian cells [4,5]. Genetic analyses showed that yeast cells lacking CCH1 or MID1 and those lacking both genes have the same phenotype, including low Ca\textsuperscript{2+} uptake activity and loss of viability after exposure to the mating pheromone α-factor [1,2]. It is hypothesized that the Cch1 and Mid1 proteins are necessary constituents of a common Ca\textsuperscript{2+} influx system [1,2,6–9], and this is supported by the observation that Mid1 co-immunoprecipitates with Cch1 [6]. However, it cannot be proven by gene disruption analysis that these two proteins are sufficient for the formation of the Ca\textsuperscript{2+} influx system. To overcome this obstacle, molecular cloning of the encoding genes and consequent in vivo or in vitro reconstitution experiments are essential. Unfortunately, the molecular cloning of CCH1 is not possible using conventional techniques with Escherichia coli, because the CCH1 sequence is harmful to this organism [2].

In S. cerevisiae, the ends of DNA molecules are extremely recombinogenic [10], allowing for targeted recombination between two DNA molecules which have homologous sequences at their ends. Based upon this property, in vivo cloning techniques have been developed that co-transform a linearized vector with a gene of interest [11–13]. In the present study, we employed this technique to clone the CCH1 gene in a cch1 mutant and found that the cloned gene complements the mutation, thereby indicating that it is functional. A further study showed that overexpression of CCH1 or MID1 from a multicopy plasmid does not increase Ca\textsuperscript{2+} uptake activity, but co-overexpression induces a 2- to 3-fold increase in growing and mating pheromone-treated cells, suggesting that overproduction of Cch1 and Mid1 is sufficient to increase Ca\textsuperscript{2+} uptake activity. The co-overexpression system is useful for functional analysis of animal, plant and fungal Ca\textsuperscript{2+}-permeable channel candidates and their regulators.

2. Materials and methods

2.1. Yeast strains and media

The yeast genetic manipulation and recombinant DNA techniques were previously described [14,15]. E. coli strain DH5α was used (Toyobo Co., Osaka). The isogenic wild-type yeast strains used were H207 (MATa his3::Δ LEU2-3,112 TRP1-289 URA3-52 SSt1-2) and H208 (MATa his3::Δ LEU2-3,112 TRP1-289 URA3-52 SSt1-2) [3]. The following mutant strains are derivatives of H207: H311 (MATa mid1-Δ::HIS3) [16], H313 (MATa cch1Δ::TRP1) and H315 (MATa cch1Δ::TRP1 mid1-Δ::HIS3). To construct H312, H313 and H315, the CCH1-knockout plasmid pKC299 (a gift from K.W. Cunningham) [6] was cut with EcoRI and introduced into H208. Successful disruption of the CCH1 gene was confirmed by PCR and tetrad analysis. The resulting strain, H314 (MATa cch1Δ::TRP1), was crossed with H311 (MATa mid1-Δ::HIS3), and the diploids formed were sporulated and dissected. A Trp+ His+ segregant (MATa mid1-Δ::HIS3) was designated...
H312, a Trp⁺ His⁺ segregant (Matα cch1Δ:TRP1) was designated H313, and a Trp⁺ His⁺ segregant (Matα cch1Δ:TRP1 mid1Δ:His3) was designated H315.

Rich medium YPD and synthetic media SD and SD+Ca100 were prepared as previously described [3]. The SD+Ca100 medium contained 100 μM CaCl₂, while SD medium contained 681 μM CaCl₂.

2.2. Plasmids

Yeast shuttle vectors YCplac33 [CEN4 URA3], YEpCacl11 [CEN4 LEU2], YEpC195 [2 μm URA3] and YEpEacl81 [2 μm LEU2] were gifts from Akio Sugino [7, 17]. YCpMID1 [CEN4 URA3 MID1], YEp-MID1 [2 μm URA3 MID1] and YEp-MID[2 μm LEU2 MID1] were constructed by inserting the MID1-containing BamHI–Xhol fragment of YCPMID1-23 [3] between the BamHI and SalI sites of YCPplac33, YEpC195 and YEpEacl81, respectively. To construct YCp33Rec [CEN4 URA4] and YEp195Rec [2 μm URA3], the vectors used for in vivo homologous recombination, the Rec sequence, a 105 bp EcoRI-BamHI fragment of pGADT7-Rec (BD Biosciences Clontech, Palo Alto, CA) was inserted between the EcoRI and BamHI sites located in the multi-cloning site (MCS) of YCplac33 and YEpC195, respectively. The Rec sequence has a SmaI site 40 bp downstream of the EcoRI site.

2.3. Cloning of CCH1

The PCR primers used are listed in Table 1. To clone the CCH1 gene by in vivo homologous recombination, it was amplified by PCR with LA Taq DNA polymerase (Takara Bio Inc., Otsu, Shiga) using primers No. 1 and No. 2 (Table 1), which possess a part of the CCH1 non-coding region and vector sequence, and chromosomal DNA of the wild-type strain H207 as a template (see Fig. 1). The PCR products were mixed with linearized YCp33Rec or YEp195Rec, which had been cut with EcoRI and SmaI, and co-introduced into strain H313 (cch1Δ:TRP1). The resulting transformants were examined for the presence of the CCH1 gene and flanking primer sequences by PCR analysis with Ex Taq DNA polymerase (Takara Bio Inc.), and sequence-positive transformants were further examined for viability 8 h after the addition of α-factor. If the construction of CCH1-carrying plasmids was successful, the transformants were viable like wild-type strains because of the presence of functional CCH1. Viable strains were selected and single colony isolation was repeated twice. The confirmed strains were designated H313/YCpCCH1Rec and H313/ YEpCCH1Rec.

Table 1

<table>
<thead>
<tr>
<th>No.</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>CCH1-5-500</td>
<td>5'-GGGTCGACCTGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>2</td>
<td>CCH1-3-142</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>3</td>
<td>CCH1-3-500-FKp</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>4</td>
<td>CCH1-5-F-Bam</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>5</td>
<td>CCH1-5-F-Pst</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>6</td>
<td>CCH1-5-F-Bam</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>7</td>
<td>CCH1-3-500-Sal</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>8</td>
<td>CCH1-3-500-Xho</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
<tr>
<td>9</td>
<td>CCH1-3-500-Pst</td>
<td>5'-GGGCATGCGCATTTTTTTCTTTTCTTTTTTCCC-3'</td>
</tr>
</tbody>
</table>

2.5. Fluorescence microscopy and immunoblotting

Fluorescence microscopy on cells expressing Cch1-GFP or Mid1-GFP was performed as described previously [16]. Immunoblot analysis was carried out according to the method described previously [3] except that SDS-PAGE samples were heated for 3 min at 70 °C. A mouse monoclonal antibody against the HA-tag (12CA5, Babco, Berkeley, CA) and affinity-purified rabbit antibodies against the C-terminal peptide of Mid1 [19] were used to detect Cch1-HAx4 and Mid1, respectively.

2.6. Determination of Cch1-GFP and Cch1-HAx4

By constructing plasmids carrying genes encoding Cch1 tagged with green fluorescence protein (GFP) or with four times-repeated hemagglutinin (HAx4) at the C-terminus of Cch1 by in vivo homologous recombination, we first constructed plasmids (named GFP-plus plasmids) that carried the 5'-non-coding (500 bp), 5'-coding (72 bp) and 5'-coding (67 bp) regions of CCH1, the tag (GFP or HAx4), and the 3'-non-coding region (142 bp) of CCH1, in which each tag was located just before the stop codon of CCH1. These regions were amplified by PCR with LA Taq DNA polymerase and the following sets of primers: for the amplification of the 5'-non-coding region, the primers No. 3 and No. 4 were used; for the 3'-non-coding region, No. 5 and No. 6; for the 5'-coding region, No. 7 and No. 8; and for the 3'-coding region, No. 9 and No. 10. As a template, DNA prepared from H313/YEpCCH1Rec was used. The resulting PCR products were cut with the appropriate restriction enzymes and inserted stepwise into YCPplac33 and YEpEacl81. The GFP sequence amplified with primers No. 11 and No. 12 and the template plasmid pS65T-C1 (BD Biosciences Clontech) followed by digestion with SalI and PstI was inserted into the resulting plasmids cut with SalI and PstI to give the GFP-plus plasmids. Likewise, the HAx4 sequence obtained from pLSK-cof-HA1 [18] was inserted into the resulting plasmids to give the HAx4-plus plasmids. The GFP-plus and HAx4-plus plasmids were linearized with XhoI and introduced into cch1Δ cells with the CCH1 sequence amplified by PCR using primers No. 7 and No. 10 and chromosomal DNA of the wild-type strain as a template. The resulting transformants were then examined by PCR and cell viability was assessed to determine whether it contained plasmids carrying the fusion genes encoding functional Cch1-GFP or Cch1-HAx4. Successful plasmids were designated YCpCCH1-GFP, YCpCCH1-HAx4, YEpCCH1-GFP and YEpCCH1-HAx4.

2.7. Determination of mids and viability

The methods used were previously described [3].
3. Results and discussion

3.1. Cloning of CCH1 by in vivo homologous recombination

Since several research groups including ours could not clone the CCH1 gene by conventional cloning methods using E. coli as a host [2], we employed another method using in vivo homologous recombination in S. cerevisiae. The CCH1-open reading frame (ORF) with 5’-500 bp and 3’-142 bp flanking regions fused with sequences homologous to the ends of linearized vector YEp195Rec [URA3] (low-copy) and YEp195Rec [URA3] (multi-copy) was amplified by PCR using primers No. 1 and No. 2 listed in Table 1. The 5’-primer (No. 1) contained the distal 27 nucleotides of the 5’-flanking region and a part of the vector sequence, and the 3’-primer (No. 2) contained the distal 27 nucleotides of the 3’-flanking region and a part of the Rec sequence in the vectors. The PCR products and the linearized YEp195Rec or YEp195Rec were co-introduced into the yeast cells, in which homologous recombination occurred to produce a plasmid containing the CCH1 gene. The PCR primer (No. 2) contained the initiation codon; T, termination codon; ORF, open reading frame; E, EcoRI site; S, SmaI site; B, BamHI site.

Fig. 1. Scheme of the cloning of the CCH1 gene by in vivo homologous recombination in yeast. The CCH1 gene was amplified by PCR using primers containing sequences homologous to a linearized plasmid at both ends. The PCR product was mixed with the linearized plasmid and co-transformed into yeast cells, in which homologous recombination occurred to produce a plasmid containing the CCH1 gene. Abbreviations used: I, initiation codon; T, termination codon; ORF, open reading frame; E, EcoRI site; S, SmaI site; B, BamHI site.

3.2. Comparison of CCH1 and MID1 genes

The CCH1 and MID1 genes are applicable for yeast cells. Therefore, we employed another method using in vivo homologous recombination in yeast. The CCH1 gene cloned in yeast cells. Therefore, we employed another method using in vivo homologous recombination in yeast. The CCH1 gene cloned in yeast cells. Therefore, we employed another method using in vivo homologous recombination in yeast. The CCH1 gene cloned in yeast cells. Therefore, we employed another method using in vivo homologous recombination in yeast.

Fig. 2. Ca2+ accumulation of cells overexpressing CCH1, MID1 or both in the exponentially growing phase. Exponentially growing cells of WT, CCH1ox, MID1ox, and CCH1ox MID1ox strains in SD.Ca100 medium received 185 kBq/ml (1.8 kBq/nmol) 45CaCl2 at time 0 and were then examined for Ca2+ accumulation at the indicated times. Ca2+ accumulation is shown as a function of time (A) and generation (B) as the abscissa axis. Data are means ± S.D. from at least three independent experiments. In (A), statistical analysis showed that Ca2+ accumulation in CCH1ox MID1ox cells was significantly greater than that in WT, CCH1ox, and MID1ox cells (P < 0.05).
It appeared that YEpCCH1Rec and YCpCCH1Rec did not replicate in E. coli. The plasmids were isolated from H313/YEpCCH1Rec and H313/YCpCCH1Rec strains and introduced into E. coli by conventional transformation techniques. However, no transformant was obtained.

To transfer YEpCCH1Rec to the wild-type strains, H313/YEpCCH1Rec was crossed with a MATa wild-type strain, H208, and the resulting diploids were sporulated and dissected. From the segregants, MATa wild-type strains carrying YEpCCH1Rec, designated H207/YEpCCH1Rec, were selected and used in further experiments. Hereafter, we call cells of the H207/YEpCCH1Rec strain CCH1ox cells. Likewise, we call H207/YEpMID1 cells MID1ox cells, H207 cells bearing both YEpCCH1Rec and YEpMID1 CCH1ox MID1ox cells, and H207 cells bearing the corresponding empty vectors WT cells unless otherwise mentioned.

3.2. CCH1ox cells result in cell cycle retardation

CCH1ox cells grew more slowly in liquid and solid media than WT cells. Therefore, we determined the mean doubling time (TD) of cells grown in SD Ca100 medium at 30 °C. The TD of CCH1ox cells was 4.2 ± 0.2 h, whereas that of MID1ox and WT cells was 3.0 ± 0.2 h. The TD of CCH1ox MID1ox cells was 5.0 ± 0.3 h. Therefore, overexpression of CCH1, but not MID1, retards cell cycle progression and co-overexpression of CCH1 and MID1 has an additional effect on cell cycle progression.

3.3. Co-overexpression of CCH1 and MID1 increases Ca2+ uptake activity in growing cells

We examined the effect of overexpression of CCH1 on Ca2+ uptake activity. Cells of various strains were grown in SD Ca100 medium to the exponentially growing phase and incubated further with 45CaCl2, after which Ca2+ uptake activity was measured. Since TD varies from strain to strain, the data obtained were plotted against generation time as well as incubation time (Fig. 2A and B). In all the strains examined, only CCH1ox MID1ox cells showed elevated activity for the initial Ca2+ uptake rate and longer term Ca2+ accumulation. The Ca2+ uptake activity of CCH1ox cells was unchanged, although cell cycle progression was retarded in this strain. These results suggest that a Ca2+ channel in growing cells requires the Cch1 and Mid1 proteins for their functionality. In addition, cell cycle retardation caused by overexpression of CCH1 is not due to increased Ca2+ influx.

3.4. Co-overexpression of CCH1 and MID1 increases Ca2+ uptake activity in β-factor-treated cells

Previous studies with mutants lacking CCH1, MID1 or both showed that Cch1 and Mid1 co-operate in common Ca2+ influx systems including a β-factor-induced Ca2+ influx system [1,2,7]. Therefore, we examined if overexpression of CCH1 and MID1 affects cell viability and Ca2+ uptake activity after cells were exposed to β-factor. Fig. 3A shows that the viability of cells exposed to β-factor was the same among CCH1ox, MID1ox, CCH1ox MID1ox, and WT cells, except that CCH1ox MID1ox cells maintained a slightly higher viability after three generations had passed. Fig. 3B shows that among the four strains examined, only CCH1ox MID1ox cells possessed enhanced activity in initial Ca2+ uptake rate and longer term Ca2+ accumulation. In contrast, CCH1ox cells did not exhibit increased activity. MID1ox cells possess no elevated activity [3]. It should be noted that overexpression of CCH1, MID1 or both did not affect dose–response curves to β-factor and kinetics for differentiation into cells having a mating type [3].

3.5. Overexpression of CCH1 does not complement the mid1Δ mutation

Molecular cloning of CCH1 on a multicopy plasmid in yeast allowed us to test whether CCH1 can complement phenotypes of the mid1Δ mutant when overexpressed. Exponentially growing cells of H311/YEpCCH1Rec (mid1ΔCCH1ox) were incubated with β-factor, and cell viability and Ca2+ uptake activity were determined at appropriate intervals. Fig. 4A shows that mid1ΔCCH1ox cells did not have a significantly higher viability than mid1Δ cells. In addition, Ca2+ uptake activity was the same between mid1ΔCCH1ox and mid1Δ cells (Fig. 4B). These results indicate that overexpression of CCH1 does not complement the mid1Δ mutation.

Fig. 4C shows that there was no significant difference in viability and Ca2+ uptake activity between cch1Δmid1Δ cells.
as for Mid1, CCH1. This indicates that overexpression of dilute sample from the multicopy plasmid was still detectable. from the low copy plasmid was barely detectable, a 32-fold-

increase in protein content. As for Mid1, overexpression of MID1 also resulted in an at least 32-fold increase compared to the endogenous content (Fig. 5B).

It is possible that excess Cch1 produced from the multicopy plasmid was mostly mislocalized. To investigate this possibility, the Cch1-GFP protein was produced from low copy and multicopy plasmids and subjected to fluorescence microscopy. Cch1-GFP produced from the low copy plasmid was barely observed (Fig. 6A), while that from the multicopy plasmid was clearly seen in the plasma membrane (Fig. 6B). Similar fluorescent images were obtained with Mid1-GFP (Fig. 6E and F). It is noted that both Cch1-GFP and Mid1-GFP were also localized in an intracellular compartment. As for Mid1 and Mid1-GFP, this compartment is shown to be the endoplasmic reticulum (ER), and localization to the ER is not due to overexpression from multicopy plasmids [16,19].

These data suggest that the 2- to 3-fold-increase in Ca²⁺ uptake activity is not attributable to a small increase in Cch1 or Mid1 content or the mislocalization of Cch1 or Mid1. Why is the increase in Ca²⁺ accumulation only 2-3-fold in CCH1ox MID1ox cells irrespective of these facts? We suppose that it is due to the presence of a cellular factor responsible for the assembly or functional regulation of Cch1 and Mid1, and that this factor is limiting in CCH1ox MID1ox cells. Further studies are needed to identify such a factor. In the mating process, an α-factor-inducible, cycloheximide-sensitive factor has been suggested as a candidate [21].

It should be noted that measurements of Ca²⁺ accumulation, the method we employed, do not necessarily point to an increase in Ca²⁺ channel activity in CCH1ox MID1ox cells and may detect changes in Ca²⁺ transport through other means. However, considering that Cch1 is structurally homologous to mammalian voltage-gated Ca²⁺ channels [1,2] and Mid1 has stretch-activated Ca²⁺-permeable channel.
activity [4,5], we believe that at least the increase in the initial Ca\(^{2+}\) accumulation rate reflects an increase in Ca\(^{2+}\) channel activity. Electrophysiological analysis is needed to clarify this matter.

If Cch1 acts as a voltage-gated Ca\(^{2+}\) channel, how is it activated in exponentially growing cells and those exposed to \(\alpha\)-factor? We have no data to answer this question at present, but speculate that expansion of the plasma membrane accompanied with budding and mating projection formation might activate Mid1. The activated Mid1 might depolarize the plasma membrane to activate Cch1.

In summary, while previous studies with the cch1, mid1 and cch1 mid1 mutants have shown that Cch1 and Mid1 are necessary components for Ca\(^{2+}\) influx [1,2,7], our overexpression experiments with the cloned CCH1 and MIDI genes clearly indicate that they are sufficient components. The CCH1ox and MIDIox strain may serve as a new tool to explore factors or subunits that upregulate or downregulate the Cch1/Mid1 channel.

Finally, we would like to highlight the usefulness of the present study. Many eukaryotic putative channels have been cloned but not functionally proven to be channels (for example, see [22]). The expression of such channels with and without Mid1 in the yeast strains described here may solve this problem.

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References


Fig. 6. Subcellular localization of Mid1 and Cch1 in exponentially growing cells. Exponentially growing cells of the cch1Δ mutant H313 harboring YCpCCH1-GFP (A, C) or YEPCCH1-GFP (B, D) and the mid1Δ mutant H311 harboring YCpMIDI-GFP (E, G) or YEPMID1-GFP (F, H) in SD.Ca100 medium were observed by confocal fluorescence microscopy (A, B, E and F) or differential interference contrast microscopy (C, D, G and H). Similar results were obtained for two additional experiments.