INTRODUCTION

Voltage-gated calcium channels (VGCCs) in animals sense a membrane potential change and drive a transient and rapid increase in cytosolic free Ca\(^{2+}\) concentrations (Catterall, 2000). VGCCs are composed of the pore-forming subunit \(\alpha_1\) and auxiliary subunits \(\beta\) and \(\gamma\), which play prominent roles in the regulation of traffic, expression and function of the \(\alpha_1\) subunit (Catterall et al., 2005; Jarvis & Zamponi, 2007). The yeast Saccharomyces cerevisiae CCH1 gene encodes a homologue of the pore-forming \(\alpha_1\) subunit of mammalian voltage-gated calcium channels. Cch1 cooperates with Mid1, a candidate for a putative, functional homologue of the mammalian regulatory subunit \(\alpha_2\delta\), and is essential for Ca\(^{2+}\) influx induced by several stimuli. Here, we characterized two mutant alleles of CCH1, CCH1* (or CCH1-star, carrying four point mutations: V49A, N1066D, Y1145H and N1330S) and cch1-2 (formerly designated mid3-2). The product of CCH1* displayed a marked increase in Ca\(^{2+}\) uptake activity in the presence and absence of \(\alpha\)-factor, and its increased activity was still dependent on Mid1. Mutations in CCH1* did not affect its susceptibility to regulation by calcineurin. In addition, not only was the N1066D mutation in the cytoplasmic loop between domains II and III responsible for the increased activity of Cch1*, but also substitution of another negatively charged amino acid Glu for Asn 1066 resulted in a significant increase in the Ca\(^{2+}\) uptake activity of Cch1. This is the first report of a hyperactive mutation in Cch1. On the other hand, the cch1-2 allele possesses the P1228L mutation located in the extracellular S1–S2 linker of domain III. The Pro\(^{1228}\) residue is highly conserved from fungi to humans, and the P1228L mutation led to a partial loss in Cch1 function, but did not affect the localization and expression of Cch1. The results extend our understanding of the structure–function relationship and functional regulation of Cch1.
because of structural features, such as N-glycosylation, a cysteine-rich domain and a putative N-terminal signal peptide (Martin et al., 2011). Deletion of either CCH1, MID1 or both results in quantitatively identical decreases in cell viability and Ca\(^{2+}\) influx following exposure to mating pheromone (Paidhungat & Garrett, 1997; Fischer et al., 1997), suggesting that Cch1 and Mid1 cooperate in the same process. This suggestion has been further supported by the finding that overexpression of both Cch1 and Mid1, but not single overexpression of either one of them, is effective in increasing Ca\(^{2+}\) influx activity (Iida et al., 2004). Muller et al. (2001) postulated that Cch1 and Mid1 constitute a high-affinity Ca\(^{2+}\) influx system that becomes functional when cells are incubated in low-Ca\(^{2+}\) media. Recently, Ecm7, a member of the PMP-22/EMP/Mid1 constitute a high-affinity Ca\(^{2+}\) influx system (Martin et al., 2011).

Cumulative works on Cch1 and Mid1 have revealed that Cch1/ Mid1 can be activated by several stimuli other than mating pheromone. These include store-operated stress (Locke et al., 2000), endoplasmic reticulum (ER) stress (Bonilla & Cunningham, 2003), hyperosmotic stress (Matsumoto et al., 2002), alkaline stress (Viladevall et al., 2004), cold stress (Peiter et al., 2005), oxidative stress (Popa et al., 2010) and ethanol stress (Cournchene et al., 2011).

The activity of Cch1 is suggested to be important for the activation of calcineurin, a Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase, which is necessary for cellular responses to various stimuli (Bonilla et al., 2002; Miyakawa & Mizunuma, 2007; Stie & Fox, 2008).

Interestingly, activated calcineurin appears to dephosphorylate Cch1, thereby inducing the feedback-inhibition of Ca\(^{2+}\) influx in response to mating pheromone and ER stress (Bonilla & Cunningham, 2003). Also, the mitogen-activated protein (MAP) kinase Mpk1 and its upstream regulator Bck1 are specifically required for the activation of Cch1 during ER stress (Bonilla & Cunningham, 2003). Some unknown targets of transcription factor Ste2, which is activated by the MAP kinase Fus3, are necessary for Cch1 activation in response to mating pheromone (Muller et al., 2001). However, the molecular mechanisms underlying the regulation of Cch1 remain elusive. In addition, the structure–function relationship of Cch1 has not yet been investigated.

Here, to investigate the structure–function relationship, we identified two mutant alleles of CCH1, CCH1* (or CCH1-star, resulting in a higher Ca\(^{2+}\) uptake activity of the gene product) and cch1-2, formerly designated mid3-2 (Iida et al., 1994). We found that the product of the CCH1* allele had four mutations, one of which (N1066D) accounted for its increased activity. The Asn\(^{1066}\) residue is located in the cytoplasmic loop between domains II and III. This is the first report about a hyperactive mutation in Cch1. The cch1-2 allele was found to have the P1228L mutation, which leads to a partial loss in Cch1 function. Pro\(^{1228}\) is conserved completely from fungi to humans and is located in the extracellular S1–S2 linker of domain III. We discuss the importance of the structural regions containing the two mutations.

**METHODS**

**Yeast stains, plasmids and media.** The wild-type strain H207 (MATa his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2) and its derivative mutant strains H3032 [MATa cch1-2 (formerly mid3-2) his3-Δ1,112 trp1-289 ura3-52 sst1-2], H317 (MATa cch1Δa::HIS3 his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst2-1), H319 (MATa mid1Δa::HIS3 cch1Δa::HIS3 CNB1 his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2), H321 (MATa MID1 CCH cnb1Δa::HIS3 his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst1-2) and H345 (MATa mid1Δa::HIS3 cch1Δa::TRP1 cnb1Δa::HIS3 his3-Δ1 leu2-3,112 trp1-289 ura3-52 sst2-2) were used in this study.

The low-copy plasmids YCplac33, pBC111, pBCS-CCH1 and pBCT-CCH1 used in this study were described previously (Iida et al., 2007; Teng et al., 2008). Note that the wild-type CCH1 gene has been derived from yeast strain H207 and is expressed from its own promoter on pBCS-CCH1 and a strong TDI3 promoter on pBCT-CCH1. In our previous paper (Iida et al., 2007), we added the suffix ‘H’ to the H207-derived CCH1 gene, as in CCH1H, because we needed to use this suffix to distinguish between H207- and X2180-1A-derived CCH1 genes. In this paper, however, we did not add this suffix because we only used the H207-derived CCH1 gene. The low-copy plasmid YCpS-MID1 (ARS1 CEN4 URA3 MID1p-MID1-ADH1) contains the MID1 gene, expression of which is under the control of its own promoter.

Synthetic media SD and SD.Ca100 medium were prepared as described previously (Iida et al., 1994). SD.Ca100 contains 100 μM CaCl\(_2\) and SD medium 681 μM CaCl\(_2\).

**DNA sequencing and plasmid construction.** The cch1-2 (or mid3-2) gene of H3032 was amplified by PCR and was directly sequenced with the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems), as described previously (Iida et al., 2007).
**In vitro site-directed mutagenesis.** In vitro site-directed mutagenesis was performed using the QuikChange Site-directed Mutagenesis kit (Stratagene). Plasmids containing mutant CCH1 genes were as follows: pBCS-CCH1V49A, pBCS-CCH1N1066, pBCS-CCH1T1145H, pBCS-CCH1N1330S, pBCS-CCH1N1066Q, pBCS-CCH1N1066H, pBCS-CCH1N1066K and pBCS-CCH1N1066E. PCR primers used here are listed in Table 1.

**Determination of cell viability.** The viability of cells exposed to 6 μM α-factor in SD.Ca100 medium was determined by the methylene blue liquid method (Iida et al., 1990, 1994).

**Determination of Ca2+ accumulation in yeast cells.** Exponentially growing cells in SD.Ca100 medium were incubated for 2 h with 45CaCl2 (185 kBq ml⁻¹; 1.85 kBq mmol⁻¹; PerkinElmer) in the presence or absence of 6 μM α-factor. Samples were taken, 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 with a scintillation counter. Western blotting and fluorescence microscopy. Western blotting was carried out according to Iida et al. (1994) except that samples for SDS-PAGE were denatured for 15 min at 37 °C. Affinity-purified rabbit polyclonal antibodies against the Cch1 carboxyl-terminal peptide spanning from amino acid residue 1949 to 2039 described previously (Iida et al., 2007) were used at a concentration of 0.07 μg ml⁻¹ to detect wild-type and various mutant forms of the Cch1 protein. Fluorescence microscopy on cells expressing Cch1-P1228L-EGFP and Cch1-EGFP was performed as described previously (Tada et al., 2003).

**Statistical analysis.** Significance was determined using the unpaired Student’s t test with a P-value <0.05 required for significance.

## RESULTS

**CCH1* is a hyperactive mutant.**

When we cloned the CCH1 gene by PCR with LA Taq DNA polymerase with a relatively low fidelity, we acquired a mutant gene whose product had an increased Ca2⁺ uptake activity, and named it CCH1*. When exposed to the mating pheromone α-factor, cch1-defective mutants die because of a deficiency in Ca2⁺ uptake. This conditionally lethal phenotype and the low Ca2⁺ uptake ability were used to assess the activity of wild-type and mutant Cch1 proteins in this study. To characterize Cch1*, low-copy plasmids containing CCH1* or wild-type CCH1, expression of which was under the control of the CCH1 promoter, were introduced into cch1Δ cells, and the resulting transformants, designated CCH1* cells and CCH1 cells hereafter, respectively, were subjected to 4Ca2⁺ accumulation assays. Cells were incubated to the exponential phase in SD.Ca100 medium and then incubated for a further 2 h with 6 μM α-factor, a Cch1 stimulator. Results showed that CCH1 cells displayed the same Ca2⁺ uptake activity as cells expressing wild-type Cch1 from its chromosomal locus, designated WT cells hereafter [66±3.5 pmol (10⁶ cells)⁻¹ for CCH1 cells, 66±15 pmol (10⁶ cells)⁻¹ for WT cells] (Fig. 2a). Under the same conditions, CCH1* cells exhibited a significant increase in Ca2⁺ accumulation activity [108±12 pmol (10⁶ cells)⁻¹] (Fig. 2a). The amounts of Cch1* and wild-type Cch1 proteins in the respective transformants were almost the same, but were slightly lower than that of the wild-type Cch1 protein produced from its chromosomal locus, as revealed by Western blotting (Fig. 2b). Therefore, CCH1* is a hyperactive mutant. DNA sequencing of the mutant gene showed that it contained four amino acid substitutions, V49A, N1066D, Y1145H and N1330S (Fig. 1).

Cch1 has basal Ca2⁺ influx activity under non-stimulating conditions, and is activated by a variety of stimuli, including α-factor (Fischer et al., 1997; Muller et al., 2001; Paidhungat & Garrett, 1997). Therefore, we examined the Ca2⁺ influx activity of Cch1* under non-stimulating conditions. Fig. 2(a) shows that Ca2⁺ accumulation in CCH1* cells was higher than that in CCH1 cells and WT cells [57±12 for CCH1*, 16±1 for CCH1 and 15±4 for WT, in pmol (10⁶ cells)⁻¹]. This indicates that Cch1* undergoes a spontaneous increase in basal Ca2⁺ influx activity. Note that the net increase of Ca2⁺ influx activity in CCH1* cells, defined as the activity in the presence of α-factor minus the activity in the absence of it, was comparable to that in CCH1 cells (Fig. 2a). This suggests that the increased activity of CCH1* cells is not ascribed to a change in sensitivity to α-factor. To

### Table 1. PCR primers used in this study

Mutated nucleotides are underlined.

<table>
<thead>
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<tr>
<td>1066Gln-R</td>
<td>5’-CATATTCGCGACTTTTGAATTACGGCGGTTTCTC-3’</td>
</tr>
<tr>
<td>1066His-F</td>
<td>5’-GAGGAACCCGATATGCATATACGGCGAGATATG-3’</td>
</tr>
<tr>
<td>1066His-R</td>
<td>5’-CATATTCGCGACTTTTGCAATTTGCGGTTTCTC-3’</td>
</tr>
<tr>
<td>1066Lys-F</td>
<td>5’-GAGGAACCCGATATGGAATTACGGCGAGATATG-3’</td>
</tr>
<tr>
<td>1066Lys-R</td>
<td>5’-CATATTCGCGACTTTTGCAATTTGCGGTTTCTC-3’</td>
</tr>
<tr>
<td>1066Glu-F</td>
<td>5’-GAGGAACCCGATATGGAATTACGGCGAGATATG-3’</td>
</tr>
<tr>
<td>1066Glu-R</td>
<td>5’-CATATTCGCGACTTTTGCAATTTGCGGTTTCTC-3’</td>
</tr>
</tbody>
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examine this suggestion further, we performed dose–response experiments with this factor. Fig. 2(c) shows that the Ca$^{2+}$ influx activities of CCH1 and CCH1* cells were increased roughly in parallel with the increase of the α-factor concentration we tested, supporting the above suggestion.

**Increased activity of Cch1* depends on Mid1**

As Cch1 functions with Mid1 to mediate Ca$^{2+}$ influx in α-factor-stimulated cells (Fischer et al., 1997; Iida et al., 2004; Paidhungat & Garrett, 1997), this raises the question of whether the increased activity of Cch1* still depends on Mid1. To address this, we introduced a plasmid containing CCH1* into mid1Δ cch1Δ cells to obtain transformants (designated mid1Δ CCH1* cells). We selected transformants producing the Cch1* protein whose amount was essentially the same as that of the Cch1 protein produced by WT cells (data not shown) and those selected were subjected to Ca$^{2+}$ uptake assays in the presence and absence of α-factor. As shown in Fig. 2(a), when the MID1 gene is deleted, the higher activity of Cch1* decreased dramatically to a level comparable to that of cch1Δ cells in both the presence and the absence of α-factor. As shown in Fig. 2(a), when the MID1 gene is deleted, the higher activity of Cch1* decreased dramatically to a level comparable to that of cch1Δ cells in both the presence and the absence of α-factor. [10 ± 7 for mid1Δ CCH1* versus 17 ± 1 for cch1Δ in the presence of α-factor; 3 ± 1 for mid1Δ CCH1* versus 3 ± 1 for cch1Δ in the absence of α-factor; in pmol (10$^6$ cells)$^{-1}$]. These results indicate that the higher activity of Cch1* is dependent on Mid1.

**Cch1* maintains its susceptibility to regulation by calcineurin**

As Cch1 activity is negatively regulated by calcineurin (Bonilla & Cunningham, 2003), we presumed that the increased activity of Cch1* may be caused by loss or attenuation of negative regulation by calcineurin. To examine this possibility, we investigated the Ca$^{2+}$ uptake activity of cnb1Δ cch1Δ mid1Δ cells co-expressing CCH1* and MID1 (cnb1Δ CCH1*) and that of cnb1Δ cells carrying the respective empty vectors (cnb1Δ). Cnb1 is the regulatory subunit of calcineurin, and deletion of CNB1 results in a lack of calcineurin activity (Cwert & Thörner, 1992; Liu et al., 1991). The amount of Cch1* produced from the CCH1 promoter in cnb1Δ CCH1* cells was comparable to that of cnb1Δ cells (Fig. 3b). Two hours after exposure to α-factor, Ca$^{2+}$ uptake activity in cnb1Δ cells was approximately sevenfold higher than that in WT cells (455 ± 62 for cnb1Δ versus 66 ± 15 for WT, pmol/10$^6$ cells) (Figs 2a, 3a), as reported previously (Bonilla & Cunningham, 2003). Ca$^{2+}$ uptake activity in cnb1Δ CCH1* cells was approximately eightfold higher than that in CCH1* cells [895 ± 182 for cnb1Δ CCH1* versus 108 ± 12 for CCH1*, in pmol (10$^6$ cells)$^{-1}$]. In the absence of α-factor, Ca$^{2+}$ uptake activity in cnb1Δ cells was approximately sevenfold higher than that in WT cells [106 ± 10 for cnb1Δ CCH1 versus 15 ± 4 for WT, in pmol (10$^6$ cells)$^{-1}$], and Ca$^{2+}$ uptake activity in cnb1Δ CCH1* cells was approximately sevenfold higher than that in CCH1* cells [417 ± 67 for cnb1Δ CCH1* versus 57 ± 12 for

![Fig. 2. CCH1* is a hyperactive mutation. (a) Ca$^{2+}$ uptake activity. Ca$^{2+}$ accumulation was measured after cells were incubated for 2 h with (w/; grey bars) or without (w/o; white bars) 6 μM α-factor in SD.Ca100 medium. Strains examined are as follows (from left to right): WT, strain H207 (WT) transformed with the empty vector pBC111; cch1Δ, CCH1 and CCH1*, strain H317 (cch1Δ) transformed with pBC111, pBCS-CCH1 and pBCS-CCH1*, respectively; mid1Δ CCH1*, strain H319 (mid1Δ cch1Δ) transformed with pBCS-CCH1*. Data are the mean ± SD of at least three independent experiments. (b) Protein levels. To detect the Cch1 protein, Western blotting was conducted using affinity-purified polyclonal antibodies raised against a C-terminal polypeptide of Cch1. Sources of whole-cell extracts are as follows: lanes 1 and 2, H207 (CCH1)pBC111; lanes 3 and 4, H317 (cch1Δ)pBC111; lanes 5 and 6, H317 (cch1Δ)pBCS-CCH1; lanes 7 and 8, H317 (cch1Δ)pBCS-CCH1*. Note that a thin band near Cch1 present in lanes 3 and 4 is a non-specific band. Enolase is a loading control. (c) Dose–response experiments with α-factor. Ca$^{2+}$ uptake activity of cells incubated for 2 h with appropriate concentrations of α-factor was measured as above. The strains used were the same as those in (b). Data are the mean ± SD of three independent experiments.](http://mic.sgmjournals.org)
$CCH1^*$, in pmol ($10^6$ cells)$^{-1}$. Note that deletion of $CNB1$ resulted in a similar increase ratio in $CCh1^*$ and Cch1. These results indicate that the increased Ca$^{2+}$ uptake activity of Cch1* is not due to the diminution of calcineurin regulation. In other words, mutations do not affect the susceptibility of Cch1 to regulation by calcineurin.

Negativity of substituted amino acids at Asp$^{1066}$ accounts for the increased activity of $CCh1^*$

As Cch1* has four missense mutations as described above (Fig. 1), we investigated which mutation(s) among these is responsible for its increased activity. We therefore constructed four kinds of mutant carrying each of the single mutations, V49A, N1066D, T1145H or N1330S, on a low-copy plasmid and mutants were examined for Ca$^{2+}$ uptake activity in the presence or absence of $\alpha$-factor. As shown in Fig. 4(a), only the N1066D mutation caused a significant increase in Ca$^{2+}$ uptake activity [118 ± 11 for N1066D versus 66 ± 15 for WT in the presence of $\alpha$-factor; 44 ± 9 for N1066D versus 15 ± 4 for WT in the absence of $\alpha$-factor; in pmol ($10^6$ cells)$^{-1}$]. On the other hand, the Ca$^{2+}$ uptake activity of the other mutations was the same as that of the WT [73 ± 12 for V49A, 68 ± 2 for T1145H, 75 ± 12 for N1330S versus 66 ± 15 for WT in the presence of $\alpha$-factor; 15 ± 1 for V49A, 14 ± 3 for T1145H, 16 ± 2 for N1330S versus 15 ± 4 for WT in the absence of $\alpha$-factor; in pmol ($10^6$ cells)$^{-1}$]. These results indicate that only the N1066D mutation is responsible for the increased activity of Cch1*.

To investigate what property of Asn is important for an increase in Cch1* activity, we substituted a variety of amino acids for Asn$^{1066}$ and examined the Ca$^{2+}$ accumulation activity of mutant proteins expressed in $cch1\Delta$ cells. As shown in Fig. 4(b), similar to Asp, substitution of the negatively charged Glu for Asn$^{1066}$ resulted in a marked increase in the Ca$^{2+}$ accumulation activity of cells treated for 2 h with $\alpha$-factor. In contrast, no effect on the Ca$^{2+}$ accumulation activity of cells was seen with substitution of an uncharged amino acid, Gin, a positively (but weakly) charged amino acid, His, and a positively charged amino acid, Lys, for Asn$^{1066}$ (Fig. 4b). Therefore, we concluded that substitution of negatively charged amino acids for Asn$^{1066}$ leads to elevations in Cch1 activity.

Based on a conventional transmembrane domain prediction of ion channels (Jan & Jan, 1990), Asn$^{1066}$ was predicted to be located in the large cytoplasmic loop connecting domains II and III. We compared the amino acid sequences of the loop connecting domain II and III of the VGCC family and found that the Asn residue is not conserved in the VGCCs family (data not shown).

The $cch1$-2 mutation has occurred at the highly conserved Pro$^{1228}$ of Cch1

We have shown that mid3 is allelic to $cch1$ (Iida et al., 2007) and that cells carrying the mid3-2 allele show phenotypes of mating pheromone-induced death (mid) and low Ca$^{2+}$ uptake (Iida et al., 1994). DNA sequencing of the genomic DNA of this mutant (strain H3032) showed that mid3-2 has a missense mutation, causing a Pro$^{1228}$ to Leu substitution (P1228L). We therefore renamed mid3-2 as $cch1$-2. We reported previously that the mid3-1 ($cch1$-1) mutation causes a Gly$^{1265}$ to Glu substitution (Iida et al., 2007).

Based on a conventional transmembrane domain prediction (Jan & Jan, 1990), Pro$^{1228}$ was predicted to be located in the extracellular linker connecting S1 and S2 segments of domain III (Fig. 1). To investigate the significance of this residue, we compared the amino acid sequences of the corresponding S1–S2 linkers of various VGCCs. As shown in Fig. 5, the proline residue is highly conserved from yeasts to humans, suggesting that this residue is important for the function of VGCCs.

P1228L substitution results in a hypoactive mutation

To examine quantitatively the effect of the P1228L mutation on Cch1 function, a low-copy plasmid containing the $cch1$-2
WT, w/ α-factor; CCH1* and P1228Lox, respectively, in Fig. 6 were used to transform the transcriptionally strong TDH3 allele driven under control of either the CCH1 promoter or the transcriptionally strong TDH3 promoter was constructed, and the resulting plasmids, designated P1228L and P1228Lox, respectively, in Fig. 6 were used to transform cch1Δ or WT cells. Transformants were then tested for viability and Ca2+ uptake activity following exposure to α-factor. The results showed that cch1Δ cells bearing P1228L had roughly half as much viability and Ca2+ uptake activity as WT cells, while those bearing P1228Lox had only a slightly higher Ca2+ uptake activity than those bearing P1228L. When P1228Lox was expressed in WT cells, viability and Ca2+ uptake activity decreased by approximately 20 and 40%, respectively. These results indicate that the P1228L substitution results in a hypomorphic activity and that this product has a partial dominant-negative effect on WT Cch1 when overexpressed.

**P1228L substitution does not affect the amount and subcellular localization of the mutant protein**

It is possible that the low activity of P1228L may be due to instability and/or mislocalization of the mutant protein. To examine these possibilities, we first checked the amount of mutant protein by Western blotting. As shown in Fig. 7(a), the amount of P1228L protein produced from the P1228L plasmid was essentially the same as that of the WT protein. In addition, the P1228Lox plasmid was found to produce approximately 25-fold more protein than the P1228L plasmid. Confocal fluorescence microscopic analysis of P1228L fused to green fluorescent protein (GFP) produced from the TDH3 promoter revealed that P1228L-GFP was localized at the plasma membrane and an ER-like organelle (Fig. 7b, bottom panels). This distribution is very similar to that of WT Cch1 expressed from the same promoter (Fig. 7b, top panels; also see Iida et al., 2004, 2007). Therefore, we concluded that the amount and subcellular localization of P1228L are normal and its low Ca2+ uptake activity is due to loss of activity.

**DISCUSSION**

In the present study, we characterized two cchl alleles, CCH1* and cchl-2 (mid3-2). First, we have shown that Cchl*, containing four mutations (V49A, N1066D, T1145H and N1330S), exhibits a significantly higher Ca2+ uptake activity than that in WT Cchl, not only in response to α-factor but also in the absence of any stimuli (Fig. 2). CCH1* is thus a hyperactive mutation. Among the four mutations, only the N1066D mutation was found to be responsible for the increase in its activity (Fig. 4a).

In addition, substitution of various amino acids with different chemical and structural properties for Asn1066 indicated that the negative charge of substituted amino acids such as Asp or Glu is responsible for the increase in its activity (Fig. 4a). These results suggest that Asn1066 is important for the regulation of Cchl activity. Our results have also shown that Cchl* activity is still normal and subcellular localization of P1228L are normal and its low Ca2+ uptake activity is due to loss of activity.

![Fig. 4. Substitution of negatively charged amino acid for Asn1066 results in a significant increase in Ca2+ uptake activity in Cchl.](image-url)

Ca2+ accumulation [pmol (106 cells)]

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</tr>
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<tr>
<td>N1066E</td>
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</table>

### Notes

- N1066D, N1066Q, N1066H, N1066K and N1066E indicate strain H317 (cch1Δ) transformed with plasmids pBCS-CCH1N1066D, pBCS-CCH1N1066Q, pBCS-CCH1N1066H, pBCS-CCH1N1066K and pBCS-CCH1N1066E, respectively.
- V, Val; A, Ala; N, Asn; D, Asp; T, Thr; H, His; S, Ser; Q, Gln; K, Lys; E, Glu.
- Data are the mean ± SD of at least three independent experiments.

- #, P=0.00005 (N1066D vs. WT, w/ α-factor); ##, P=0.00003 (N1066D vs. WT, w/o α-factor); ###, P=0.03328 (N1066E vs. WT, w/ α-factor).

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Gly1265 is a member of the voltage-gated ion channel family, is no exception. It has been shown that replacement of Gly1265 severely affects channel conformational changes in the channel (the open state), rearrangement of voltage sensor domains results in opening and closing (Sunami et al., 2001). Ch1, which is a member of the voltage-gated ion channel family, is no exception. It has been shown that replacement of Gly1265 with any larger amino acid residue in the S2–S3 linker of domain III perturbs the spatial arrangement of S2 and S3, resulting in loss of activity of Ch1 (Iida et al., 2007). It is possible that Asn1066 is important for the maintenance of the Ch1 closed structure, and replacement of this residue with a negatively charged amino acid may change the conformation of the cytoplasmic II–III interdomain loop, thereby making the Ch1 structure easy to open even in the absence of stimuli.

Activation of a high-affinity Ca\(^{2+}\) influx system (HACS) composed of Ch1 is regulated by calcineurin, the MAP kinase Mpk1 and its upstream regulator Bck1, and transcription factor Ste2 (Bonilla & Cunningham, 2003; Martin et al., 2011; Muller et al., 2001). It has also been shown that there are many proteins that prevent spontaneous activation of HACS (Martin et al., 2011). However, it is unknown how these factors regulate HACS, especially Ch1, and which sites in Ch1 are involved in interactions with those regulators. By contrast, the interaction sites of mammalian VGCC \(\alpha_1\) subunits are known to some extent. For example, the II–III interdomain loop of Ca\(^{2+}\) and Ca\(^{2+}\)2 subunits interacts with a number of synaptic proteins (Catterall, 2000; Spafford & Zamponi, 2003). The II–III interdomain loop of Ca\(^{2+}\)3.2 interacts with Gβ2 subunits (Wolfe et al., 2003) and is a substrate for calmodulin-dependent protein kinase (Welsby et al., 2003). Therefore, it is possible that the II–III loop in Ch1 is important for interactions with its regulators.

### Table 5

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<th>Species</th>
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substitution of negatively charged amino acids for Asn1066 in this loop may change the interaction property to activate Cch1. Alternatively, this substitution may cause the release of a negative regulator, leading to spontaneous Cch1 activation. Further experiments are needed to unravel the exact mechanism.

In this study, we have also shown that the P1228L mutation leads to a marked loss of activity in Cch1, and appears not to affect the stability and localization of the P1228L mutant protein because the amount and subcellular localization of P1228L are essentially the same as those of the WT Cch1 (Fig. 7). The Pro1228 residue is located in the extracellular S1–S2 linker of domain III and is well conserved from fungi to humans (Figs 1 and 5).

Activation of Cch1 requires Mid1 in response to many stimuli including α-factor. Mid1 is a putative analogue of the α2δ subunit of animal VGCCs. Coexpression of the α2δ subunit with the α1 subunit has been shown to modify many properties of the α1 subunit, including the acceleration of activation and inactivation kinetics (Wakamori et al., 1999). It has been suggested that more than one extracellular loop of the α1 subunit interacts with the α2δ subunit, and domain III appears to interact strongly with the α2δ subunit (Gurnett et al., 1997). As Cch1 has been shown to be associated with Mid1 by in vivo co-immunoprecipitation of P1228L are essentially the same as those of the WT Cch1 (Fig. 7). The Pro1228 residue is located in the extracellular S1–S2 linker of domain III and is well conserved from fungi to humans (Figs 1 and 5).

Activation of Cch1 requires Mid1 in response to many stimuli including α-factor. Mid1 is a putative analogue of the α2δ subunit of animal VGCCs. Coexpression of the α2δ subunit with the α1 subunit has been shown to modify many properties of the α1 subunit, including the acceleration of activation and inactivation kinetics (Wakamori et al., 1999). It has been suggested that more than one extracellular loop of the α1 subunit interacts with the α2δ subunit, and domain III appears to interact strongly with the α2δ subunit (Gurnett et al., 1997). As Cch1 has been shown to be associated with Mid1 by in vivo co-immunoprecipitation.
analysis (Locke et al., 2000), it is possible that the domain III extracellular S1–S2 linker of Cch1 is an important interaction site for Mid1, and the P1228L mutation in this linker may interfere with this interaction, leading Cch1 to partially lose its function.

In summary, we have found two important sites that are vital for Cch1 function. This research extends the understanding of the structure–function relationship and regulation of Cch1.

ACKNOWLEDGEMENTS

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REFERENCES


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