Voltage-gated Ca\(^{2+}\) channels (VGCCs) mediate the influx of Ca\(^{2+}\) that regulates many cellular events, and mutations in VGCC genes cause serious hereditary diseases in mammals. The yeast *Saccharomyces cerevisiae* has only one gene encoding the putative pore-forming \(\alpha_1\) subunit of VGCC, *CCH1*. Here, we identify a *cch1* allele producing a completely nonfunctional Cch1 protein with a Gly\(^{1265}\) to Glu substitution present in the domain III S2–S3 cytoplasmic linker. Comparison of amino acid sequences of this linker among 58 VGCC\(\alpha_1\) subunits from 17 species reveals that a Gly residue whose position corresponds to that of the Cch1 Gly\(^{1265}\) is completely conserved from yeasts to humans. Systematic amino acid substitution analysis using 10 amino acids with different chemical and structural properties indicates that the Gly\(^{1265}\) is essential for Cch1 function because of the smallest residue volume. Replacement of the Gly\(^{959}\) residue of a rat brain Ca\(\alpha_1.2\) \(\alpha_1\) subunit (rbCBl), positionally corresponding to the yeast Cch1 Gly\(^{1265}\), with Glu, Ser, Lys, or Ala results in the loss of Ba\(^{2+}\) currents, as revealed by the patch clamp method. These results suggest that the Gly residue in the domain III S2–S3 linker is functionally indispensable from yeasts to mammals. Because the Gly residue has never been studied in any VGCC, these findings provide new insights into the structure-function relationships of VGCCs.

Voltage-gated Ca\(^{2+}\) channels (VGCCs)\(^5\) in the plasma membrane mediate the influx of Ca\(^{2+}\) that serves as the second messenger of electrical signals to initiate many cellular events, including muscle contraction, neurotransmitter release, and gene expression (1). The pore-forming component of VGCCs is provided by the \(\alpha_1\) subunit, a protein of about 2000 amino acid residues (2). This subunit contains four structurally conserved domains (I–IV), each of which contains six transmembrane segments (S1–S6) and a membrane-associated loop between S5 and S6 (called the pore loop or P-loop). The voltage sensitivity of VGCCs and structurally related cation channels is conveyed by the S4 segments, which contain several positively charged residues. S2 and S3 contain conserved negative charges that are likely to interact electrostatically with the positively charged residues of S4. The S5–P-loop–S6 region forms the pore domain (see Fig. 1).

Mutations in the \(\alpha_1\) subunits resulting in structural aberrations cause hereditary diseases (called Ca\(^{2+}\) channelopathies) in mammals, such as incomplete congenital stationary night blindness, hypokalemic periodic paralysis, episodic ataxia type 2 and familial hemiplegic migraine in humans, and ataxia and seizures in mice (3–6). Most of the mutations are predicted to produce truncated \(\alpha_1\) subunits with no significant channel activity by introducing a premature stop codon or leading to aberrant splicing. Single missense mutations can also result in the production of complete or partial loss-of-function subunits and these mutation sites are likely to be restricted to amino acid residues at transmembrane segments or loops known to be important for channel activity (7–9).

A eukaryotic model organism, the yeast *Saccharomyces cerevisiae*, carries only one gene (designated *CCH1*) coding for a protein structurally homologous to the animal \(\alpha_1\) subunits of VGCCs. Physiologically, the Ch1 protein is necessary for mating pheromone-induced Ca\(^{2+}\) uptake (10–13), store-operated Ca\(^{2+}\) entry (14), endoplasmic reticulum stress-induced Ca\(^{2+}\) uptake (15), and a hyperosmotic stress-induced increase in cytosolic Ca\(^{2+}\) concentration (16).
When exposed to the mating pheromone α-factor, mutants lacking CCH1 die because of a deficiency in Ca^{2+} uptake (10, 11). This phenotype, as well as the Ca^{2+} uptake ability, was used to test the activity of wild-type and mutant Chc1 proteins in this study. To gain channel activity, Chc1 requires another protein, Mid1, as revealed by genetic analyses (10, 11, 13), Mid1 has no structural homologue in higher eukaryotes (17), but has stretch-activated Ca^{2+} channel activity when expressed in mammalian cells (18, 19). There is no orthologue of genes encoding the auxiliary subunits α_2δ, β, and γ of mammalian VGCCs in the S. cerevisiae genome.

rbCII is a member of an L-type VGCC α_2 subunit subfamily, Ca_1.2, isolated from the rat brain (20, 21), and has been used to study the activity of wild-type and mutant Cch1 proteins (22, 23). Here, we report that a mutant allele of yeast CCH1, formerly designated mid3-1 (17), has a missense mutation causing a Gly^{3265} to Glu substitution that results in a complete loss-of-function. The glycine residue is located in the cytoplasmic linker between the S2 and S3 segments in domain III, completely conserved during evolution and proven to be important as being the smallest residue volume. In addition, we show that the Gly residue is not only important for Chc1 function, but also for rbCII channel activity. Because attention has never been focused on the Gly residue in this linker of any VGCC, our findings should help elucidate the structure-function relationships of VGCCs.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The parental strain H207 (MATα his3-Δ1 leu2–3,112 trp1–289 ura3–52 sst1–2) and its derivative mutant strains H3031 (MATα cch1-1 (formerly mid3-1) his3-Δ1 leu2–3,112 trp1–289 ura3–52 sst1–2) and H314 (MATα cch1Δ::TRP1 his3-Δ1 leu2–3,112 trp1–289 ura3–52 sst1–2) were described previously (13, 17). To construct H317 (MATα cch1Δ::HIS3 his3-Δ1 leu2–3,112 trp1–289 ura3–52 sst1–2), a CCH1 knock-out plasmid pCCH1DH (see below) was cut with EcoRI and SphI and introduced into H207. Successful disruption of the CCH1 gene was confirmed by PCR analysis with Ex Taq polymerase (Takara Bio Inc., Otsu, Japan). The strains X2180-1A, A364A, KA31-1A, YPH500, and S173-6B were described previously (17, 24–26). Rich medium YPD and synthetic media SD and SD Ca_100 were prepared as described previously (17). The SD Ca_100 medium contained 100 μM CaCl_2 whereas SD contained 681 μM CaCl_2.

**DNA Sequencing**—The CCH1 gene of various strains was amplified by PCR with LA Taq polymerase (Takara Bio Inc.) using the primers 5’-200F-EcoKpn and 3’-305R-Sph, listed in Table 1. The resulting 6.6-kb DNA fragments were directly sequenced with BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

**Construction of Plasmids**—The plasmids used in this study were listed in Table 2. The promoter of CCH1 (P_CCH1, 200 bp), its ORF (CCH1 ORF, 6,120 bp), and the terminator (T_CCH1, 305 bp) were amplified by PCR with LA Taq polymerase using the genomic DNA of H207 or X2180-1A cells as a template. The PCR primers and an adaptor are listed in Table 1.

To construct pCCH1D, a pUC18-derived plasmid carrying P_CCH1 and T_CCH1, the PCR-amplified promoter and terminator fragments of the CCH1 gene of H207 were cut with appropriate restriction enzymes and inserted into the multicloning site of pUC18, then an adaptor, which contains a stop codon and an XhoI site, was inserted between P_CCH1 and T_CCH1. The CCH1 knock-out plasmid pCCH1DH was constructed by inserting the HIS3-containing 1.8-kb BamHI-XhoI fragment of pJJ215 (27) between P_CCH1 and T_CCH1 of pCCH1D.

To clone the CCH1 gene in Escherichia coli, we first constructed a new S. cerevisiae-E. coli shuttle vector (named pBC111), which replicates with a low copy number in E. coli, by replacing the ColE1 ori of YCplac111 (28) with the ColE1 ori and the rop gene of pBR322, as follows. pBR322 was cut with SalI and MscI and the overhangs were filled-in using a DNA blunting kit (Takara Bio Inc.). The larger fragment was self-ligated and the SalI site that remained was destroyed by a series of SalI-cut, fill-in, and self-ligation. The 2.9-kb AatII-SphI fragment of the resulting plasmid was ligated with the 3.9-kb SphI-AatII fragment of YCplac111 to produce pBC111. pBBS111 and pBCT111 are derivatives of pBC111, each of which carries P_CCH1-T_CCH1 or P_TDHF-T_ADHF, respectively. pBBS111 was constructed by inserting the 0.5-kb EcoRI-SphI fragment of pCCH1D into the multicloning site of pBCC111. To construct pBCT111, P_CCH1 and T_CCH1 of pBBS111 were replaced with the P_TDHF-containing 0.7-kb EcoRI-BamHI fragment of pUGPD (29) and the T_ADHF-containing 0.3-kb PstI-SphI fragment of pGBK7 (Clontech, Palo Alto, CA), respectively. The PCR-amplified CCH1 ORF derived from H207 or H3031 was cut with BamHI and Sall and inserted between the BamHI and Sall sites of pBCC111 or pBCT111. The resulting plasmids were designated pBCC-CCH1H or pBCC-CCH1Hm1 and pBCT-CCH1H or pBCT-CCH1Hm1, respectively. Recombinant Chc1 proteins expressed from these plasmids have three additional amino acid residues (Val, Asp, and Thr) at the carboxyl terminus. The plasmids, pBCT-CCH1H-EGFP or pBCT-CCH1Hm1-EGFP, were used to express EGFP-tagged Chc1 proteins were constructed by inserting the 0.6-kb NcoI (blunted)-NotI fragment of pEGFP (Clontech) between the Sall (blunted) and NotI sites of pBCT-CCH1Hm1, respectively. pBCT-CCH1Hm1, recombinant Chc1 proteins expressed from these plasmids have three additional amino acid residues (Val, Asp, and Thr) at the carboxyl terminus. The plasmids, pBCC-CCH1H-EGFP or pBCC-CCH1Hm1-EGFP, were used to express EGFP-tagged Chc1 proteins were constructed by inserting the 0.6-kb NcoI (blunted)-NotI fragment of pEGFP (Clontech) between the Sall (blunted) and Notl sites of pBCT-CCH1Hm1, respectively. pBCT-CCH1Hm1-EGFP, a low-copy derivative of the mammalian expression plasmid pCMS-EGFP (Clontech) was constructed as follows. The restriction sites spanning from SphI to XbaI of the multicloning site of pBCC111 were deleted, and the 2.9-kb AatII (blunted)-BamHI fragment of the resulting plasmid was inserted into the BglII and ApaI (blunted) sites of pCMS-EGFP. rbCII cDNA (kindly gifted by Dr. T. P. Snutch; see Refs. 20 and 21) was inserted between the MluI and Sall sites of the multicloning site of pBCCMS-EGFP. The resulting plasmid, pBCCMS-EGFP-rbCII, was used to transiently express rbCII under the control of the immediate-early promoter/enhancer of cytomegalovirus (CMV promoter) in BHK6 cells.
In Vitro Site-directed Mutagenesis—In vitro site-directed mutagenesis was performed using the two-step PCR method reported by Higuchi et al. (30). The PCR primers are listed in Table 1. pBCT111 plasmids bearing various \( \text{cch1} \) mutant genes were constructed by replacing the 0.4-kb KpnI-ApaI fragment of pBCT-CCH1H with the corresponding fragment that had been produced by PCR-based, in vitro site-directed mutagenesis, and the nucleotide sequence was verified. pBCS111 plasmids bearing the same \( \text{cch1} \) mutant genes were constructed by replacing the 4.1-kb SacI-SalI fragment of pBCS-CCH1H with the corresponding fragment of the pBCT111 bearing the \( \text{cch1} \) mutant genes constructed as described above.

In the case of rbCII, QuikChange II XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to mutagenize the rbCII cDNA subcloned in pBluescript II SK\(-\). The 1.4-kb SpeI-EcoRV fragment of the wild type cDNA was replaced with each mutated fragment, and each

### Table 1

Oligonucleotides used in this study

| Usage | Name | Sequence |
|-------|------|----------|
| Promoter | 5'-200F-EcoKpn | 5'-GGGAATTCGGTGACACCTAGGTAAAAGTCGACAGAGGAAATG-3' |
| | 5'R-Bam | 5'-GGGAATTCGGTGACACCTAGGTAAAAGTCGACAGAGGAAATG-3' |
| Terminator | 3'F-Pst | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| | 3'-305R-Sph | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| ORF | 3'F-Bam | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| | 3'R-Sal | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| Co-peptide | 5'-F-Bam | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| | 5'-3'R-Sal | 5'-GGCGCTACCTATCGTGGTTTGGCTTATTA-3' |
| Mutagenesis | 3298F | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |
| | 399R | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |
| | G1265S-F\(^{*}\) | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |
| | G1265S-R\(^{*}\) | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |
| | G959E-F\(^{*}\) | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |
| | G959E-R\(^{*}\) | 5'-CACTTAAAAGGCTGGAATGTTTGC-3' |

\(^{*}\) Asterisk. The G1265S-F and G1265S-R were used to substitute Ser for the Gly\(^{1265}\) of Cch1, and the G959E-F and G959E-R were used to substitute Glu for the Gly\(^{959}\) of rbCII, for example. The doubly underlined triplets in these oligonucleotides were changed to appropriate nucleotides to substitute the corresponding amino acid for Gly\(^{1265}\) or Gly\(^{959}\). Boldfaced indicates the changed nucleotides.

### Table 2

Plasmids used in this study

| Plasmid | Characteristics | Source |
|---------|----------------|--------|
| E. coli plasmids | | |
| pCCH1D | | |
| pCCH1DH | | |
| | PCCH1 T_CCH1 in pUC18 | This study |
| | P_CCH1 HST T_CCH1 in pUC18 | This study |
| Expression plasmids | | |
| YCplac111 | | |
| pBC111\(^{a}\) | ColE1-ori AB51 CEN4 LEU2 | Gietz and Sugino (28) |
| pBCS111 | ColE1-ori-rop in YCplac111 | This study |
| pBCS-CCH1H\(^{a}\) | CCH1H ORF in pBCS111 | This study |
| pBCS-CCH1Hm1\(^{b}\) | cch1-1 ORF in pBCS111 | This study |
| pBCT111 | CCH1H ORF in pBCT111 | This study |
| pBCT-CCH1H\(^{a}\) | CCH1H ORF in pBCT111 | This study |
| pBCT-CCH1Hm1\(^{b}\) | cch1-1 ORF in pBCT111 | This study |
| pBCT-CCH1H-EGFP\(^{a}\) | CCH1H-EGFP ORF in pBCT111 | This study |
| pBCT-CCH1Hm1-EGFP\(^{b}\) | cch1-1-EGFP ORF in pBCT111 | This study |
| pBCMS-EGFP\(^{a}\) | ColE1-ori-rop P_cmv EGFP marker | This study |
| pBCMS-EGFP-rbCII | rbCII cDNA in pBCMS-EGFP | This study |

\(^{a}\) The ColE1 ori of YCplac111 and pCMS-EGFP have been replaced with a DNA fragment containing the ColE1 ori and the rop gene derived from pBR322.

\(^{b}\) The suffixes of CCH1, i.e. H and Hm1, represent the CCH1 and cch1-1 ORFs derived from the strains H207 (CCH1) and H3031 (cch1-1), respectively.
mutated full-length cDNA was introduced into pHCMS-EGFP after the nucleotide sequence was verified.

**Preparation of Polyclonal Anti-Cch1 Antibodies**—A PCR fragment encoding the Cch1 carboxyl-terminal peptide spanning from amino acid residue 1,949 to 2,039 was cut with BamHI and SalI and inserted into pQE30 (Qiagen Inc., Valencia, CA) to be conjugated with a His6 tag at the amino terminus. The His6-tagged carboxyl-terminal peptide was purified from an *E. coli* transformant (strain JM109) carrying this plasmid using nickel-nitrilotriacetic acid-agarose beads (Qiagen Inc.) under denaturing conditions. Affinity purified rabbit antibodies against this peptide were prepared by Promega Co. (Madison, WI).

**Western Blotting and Fluorescence Microscopy**—Western blotting was carried out according to the method described previously (17) except that SDS-PAGE samples were heated for 3 min at 70 °C. The affinity purified rabbit polyclonal antibodies against the carboxyl-terminal peptide described above were used at a concentration of 0.07 μg/ml to detect the wild-type and various mutant forms of the Cch1 protein. Fluorescence microscopy on cells expressing the Cch1G1265E-EGFP and Cch1-EGFP was performed as described previously (31).

**Determination of the Viability of Yeast Cells**—The viability of cells exposed to 6 μM α-factor in SD.Ca100 medium was determined using the methylene blue liquid method (24).

**Determination of Ca\(^{2+}\) Accumulation in Yeast Cells**—Exponentially growing cells in SD.Ca100 medium were incubated for 2 h with 6 μM α-factor and 185 kBq/ml (1.85 kBq/nmol) of \(^{45}\)CaCl\(_2\) (PerkinElmer Life Sciences). Samples were taken, filtered through Millipore filters (type HA; 0.45 μm) that had been presoaked in 5 mM CaCl\(_2\), and washed five times with the same solution. The radioactivity retained on the filters was counted with a scintillation mixture, ReadyProtein (Beckman Coulter, Fullerton, CA) and A/D converter (Digidata 1200, Axon Instruments). Data acquisition was performed by using pCLAMP7 software (Axon Instruments, Foster City, CA) and A/D converter (Digidata 1200, Axon Instruments). Capacitative components were electrically compensated. Leak subtraction was performed with P/N method (N = 4 – 6). All experiments were performed at room temperature.

Ba\(^{2+}\) currents (\(I_{\text{Ba}}\)) through Ca\(^{2+}\) channels exogenously expressed in BHK6 cells were measured as described previously (22). The external solution contained 137 mM NaCl, 5.4 mM KCl, 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose, and 2 mM BaCl\(_2\) (pH adjusted to 7.4 with NaOH at room temperature). The resistance of the recording pipettes was 2–2.5 MΩ when filled with the pipette solution containing 120 mM CsMeSO\(_4\), 20 mM tetraethyl ammonium chloride, 14 mM EGTA, 5 mM Mg-ATP, 5 mM Na\(_2\) creatine phosphate, 0.2 mM GTP, and 10 mM HEPES (pH 7.3 adjusted with CsOH at room temperature).

**Immunohistochemistry**—BHK6 cells were fixed in 4% formaldehyde for 10 min, permeabilized with 1% Triton X-100 in phosphate-buffered saline, and blocked with TBS-T solution (20 mM Tris-HCl, 137 mM NaCl, 0.01% Tween 20, pH 7.4) containing 0.25 mg/ml bovine serum albumin for 1 h at room temperature. Polyclonal antibodies against Ca\(_2\)-channel 1.2 were visualized with secondary antibodies labeled with Alexa Fluor 543 or Alexa Fluor 633 (Invitrogen). Images were acquired by confocal microscopy (LSM-510 META, Carl Zeiss, Inc., Oberkochen, Germany) and presented in supplemental materials Fig. S1.

**Statistical Analysis**—Statistical significance was determined using the unpaired Student’s t test with a p value <0.05 required for significance.

## RESULTS

**Genetic Characterization of a cch1 Allele of *S. cerevisiae***—The phenotypes of the previously reported *mid3-1* recessive mutant are essentially the same as those of the *cch1Δ* mutant: these include mating pheromone-induced death, low Ca\(^{2+}\) uptake activity, and low viability in the stationary phase. In addition, the corresponding genes have not been cloned despite numerous trials (11, 13). These features prompted us to perform genetic analysis on them. The two mutants were crossed (*MATa mid3-1 X MATa cch1Δ*) and the resulting diploids showed low viability in the stationary phase, like *mid3-1* and *cch1Δ* haploids, suggesting that the two alleles belong to the same complementation group. The diploids were sporulated and subjected to tetrad analysis (32). All cells germinated from 72 spores dissected from 18 asci again showed low viability in the stationary phase. In addition, all the *MATa* cells from each spore (a total of 36 spores) showed the mating pheromone-induced death phenotype. These results indicate that *mid3-1* is allelic to *cch1Δ*. We therefore renamed *mid3-1* to *cch1-1*.

PCR products, synthesized using genomic DNA as a template of the *cch1-1* mutant (strain H3031) as well as of the parental strain H207 (CCH1), were directly sequenced. Three independent PCR products for each strain were sequenced to avoid misinterpretation due to possible PCR errors. Consequently, we identified a missense mutation (G3794A) in *CCH1* in *cch1-1*, which results in a substitution of glutamate (Glu) for glycine (Gly) at codon 1,265 (G1265E) (Table 3).

We noted a strain-dependent polymorphism in *CCH1*. Seven nucleotides and six encoded amino acids in *CCH1* of the strain

| Strain (genotype) | Amino acid position (nucleotide position) |
|------------------|------------------------------------------|
|                  | (CHH) | (CHH) |
|                  | (cch1) | (cch1) |
| H207, A364A      | 765 (2,294) Gly (GGA) Ala (GCA) Ala (GCA) |
|                  | 882 (2,646) Asn (AAT) Lys (AAY) Lys (AAY) |
|                  | 972 (2,914) Thr (ACT) Ala (GCT) Ala (GCT) |
|                  | 1,117 (3,351) Asp (GAC) Asp (GAT) Asp (GAT) |
|                  | 1,265 (3,794) Gly (GGA) Gly (GGA) Gly (GGA) |
|                  | 1,413 (4,238) Ser (AGT) Asn (AAT) Asn (AAT) |
|                  | 1,607 (4,819) Met (ATG) Val (GTG) Val (GTG) |
|                  | 1,627 (4,879) Phe (TTT) Val (GTG) Val (GTG) |

* The only mutation found in the cch1-1 allele.
H207 we determined were different from those of CCH1 of the
strain S288C listed in the Saccharomyces Genome Data base
(www.yeastgenome.org) (Table 3). Because S288C is isogenic to
a standard laboratory yeast strain, X2180-1A, we determined the
CCH1 nucleotide sequence of X2180-1A and four other
standard laboratory strains and found that H207 shares the
same nucleotide sequence with one strain and that X2180-1A
and S288C share the same sequence with three other strains
(Table 3). It is notable that the viability of X2180-1A cells was as
high as that of H207 cells after exposure to α-factor (data not
shown), suggesting that the six amino acid variations do not
affect Cch1 function.

To confirm that the G1265E mutation is the only determi-
nant for the phenotypes of the cch1-1 mutant, we first devel-
oped a new vector of low copy number in E. coli (designated
pBC111) because cloning of CCH1 has long been impossible
due to the probable toxicity of this gene or protein for E. coli
(11, 13). pBC111 is an E. coli-yeast shuttle vector having the
ColE1-ori-rop sequence ensuring 15–20 copies per E. coli
cell and an S. cerevisiae centromere ensuring 1–3 copies per
S. cerevisiae cell. Using this, we generated the G1265E muta-
tion in CCH1 cloned from X2180-1A and the resulting
mutant gene was tested for its ability to rescue the mating
phenome-induced death phenotype and low Ca\(^{2+}\) uptake
activity of the cch1Δ mutant. The results showed that this
mutant gene did not rescue the two phenotypes at all, just
like the G1265E mutant gene of H207 (data not shown), indi-
cating that the two phenotypes are attributed to the G1265E
mutation only. We therefore used the CCH1 alleles based on
the H207 background hereafter and designated the mutant
Cch1 protein with the G1265E mutation Cch1G1265E.

Gly\(^{1265}\) Is Completely Conserved—Based on a conven-
tional transmembrane domain prediction (33), Gly\(^{1265}\) was predicted
to be located in the cytoplasmic linker connecting the putative
transmembrane segments S2 and S3 of domain III (Fig. 1).
Although this residue is alternatively predicted to be included
in the S2 segment by other methods, such as SGD (www.yeast-
genome.org) and UniProt (www.expasy.uniprot.org). Here, we
followed the conventional prediction (33). To explore the
importance of the glycine residue in the domain III S2–S3
linker, we compared the amino acid sequences of the corre-
sponding linker of the VGCC family. Fig. 2 shows a multiple
amino acid sequence alignment of the linker and its neighbors
of a total of 58 VGCCs and candidates from 17 species, includ-
ing humans, yeasts, and representative model organisms, such
as mice, rats, zebrafish, fruit flies, and nematodes. This align-
ment clearly indicates that the glycine residue is completely
conserved from yeasts to humans. The finding prompted us to
investigate the importance of the Gly\(^{1265}\) of Cch1 because the
glycine residue has never been investigated in any VGCC or
candidate, although it is remarkably conserved during
evolution.

The G1265E Mutation Results in a Complete Loss of Function—
There are three possibilities for the inability of the
Cch1G1265E protein in cellular function: loss-of-function,
instability, and lack of plasma membrane targeting. To ex-
amine these possibilities, we first investigated the amount of
the Cch1 and Cch1G1265E proteins by Western blotting
and found that the amount of Cch1G1265E produced from
the cch1-1 gene was slightly smaller than that of the wild-
type Cch1 protein (Fig. 3A, left panel). However, this
decrease is unlikely to be the cause of the defect of Cch1G1265E
because an ~20-fold overproduction of Cch1G1265E under the control of a strong TDH3 promoter
(Fig. 3A, left panel) did not rescue the decreased viability and
Ca\(^{2+}\) uptake activity of the cch1Δ mutant at all (Fig. 3, B and
C). Thus, a putative instability of the Cch1G1265E protein
does not account for its inability in cellular function.

We then examined the subcellular localization of
Cch1G1265E, tagged with green fluorescence protein
(Cch1G1265E-EGFP) produced under the TDH3 promoter on
a centromere plasmid, using fluorescence microscopy and
found that Cch1G1265E-EGFP was normally localized to the
plasma membrane and the endoplasmic reticular membrane-
like structure (Fig. 3D). Note that the amount of Cch1-EGFP
and Cch1G1265E-EGFP produced under the self-promoter on
a centromere plasmid was too small to visualize their fluores-
cence images (13).

We also found that the 20-fold overproduction of
Cch1G1265E in the CCH1 strain (Fig. 3A, right panel) resulted
in only a small decrease in both the viability and Ca\(^{2+}\) uptake
activity (Fig. 3, E and F). This result is consistent with the
genetic data that the cch1-1 (mid3-1) mutation is recessive (17)
and indicates that the interferential effect of Cch1G1265E on
wild-type Cch1 is small. Taken together, we conclude that the
G1265E mutation results in a complete loss-of-function of
Cch1, not its instability and mislocalization.

Importance of Gly\(^{1265}\) as the Smallest Amino Acid Residue—We
next investigated the cause of the complete loss-of-function
of the Cch1G1265E protein. One can speculate three possi-
bilities for the cause: a negative charge, hydrophilicity, or a
large residue volume of glutamate compared with glycine.
To examine these possibilities, we systematically substituted
nine amino acids with different chemical and physical prop-
erties for Gly\(^{1265}\), introduced the resulting mutant genes on a
centromere plasmid into the cch1Δ mutant, and examined
their activity by cell viability and Ca\(^{2+}\) uptake assays (Fig. 4).
We have confirmed, with Western blotting, that the amount of the mutant proteins was comparable with that of the wild-type Cch1 when they were produced from the self-promoter, and that it was at least 15-fold greater than that of the wild-type Cch1 when produced from the TDH3 promoter (data not shown).

When the activities of the mutant proteins produced under the self-promoter were compared, the Gly-Ser and Gly-Ala substitutions reduced the Ca\textsuperscript{2+} uptake activity by \(\sim 30\%\), but did not affect cell viability (Fig. 4, A and D), indicating that the remaining \(\sim 70\%\) Ca\textsuperscript{2+} uptake activity is sufficiently high to support the viability of cells. The Gly-Asp substitution slightly reduced both the cell viability and Ca\textsuperscript{2+} uptake activity. The Gly-Asn substitution inactivated the Ca\textsuperscript{2+} uptake activity, whereas it still maintained slight viability. Finally, the Gly-Thr, Gly-Val, Gly-Gln, Gly-Leu, and Gly-Lys substitutions resulted in the complete loss of Cch1 function. It is notable that when these mutant proteins were produced under the TDH3 promoter, the viability and Ca\textsuperscript{2+} uptake activity were only slightly increased in the partially active mutant proteins and were not increased at all in the completely inactivated mutant proteins (Fig. 4, A and D), suggesting that a decrease in the amount of the mutant proteins is not a factor for the decrease in the viability and Ca\textsuperscript{2+} uptake activity.

When those results obtained with the self-promoter were re-plotted in terms of the residue volume or hydrophobicity of the amino acids, we noted a clear relationship between the residue volume and activity of the mutant proteins: Fig. 4, B and E, show that when the residue volume is \(\geq 115\) cubic Å or greater at the position of Gly1265, the Cch1 protein completely loses its activity. By contrast, Fig. 4, C and F, show that there is no relationship between hydrophobicity and Cch1 function. We therefore conclude that Gly1265 is important for Cch1 activity because of the smallest residue volume. Finally, we note that among the Gly-Asp, Gly-Asn, and Gly-Thr substitutions, three of which result in almost the same residue volume, the more the residue is
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Gly959 of Rat Ca1.2 Is Essential—On the basis of the above findings that the Gly1265 of Cch1 is essential and that the Gly residue positionally corresponding to Gly1265 is completely conserved during evolution, we examined the possibility that the Gly residue of mammalian VGCC α1 subunits is essential for their channel function. To do this, the Gly959 residue of the rat Ca1.2 (rbCII), which is the positional counterpart of the Gly1265 of Cch1, was replaced with Glu, Ser, Lys, or Ala. The resulting mutant rbCII proteins were expressed under the control of the CMV promoter in BHK6 cells expressing rabbit β1a and α2/δ subunits and examined for their function and subcellular localization.

Whole cell patch clamp recordings showed that the rbCII959E protein had no channel activity at all and that the rbCII959S protein had little activity (Fig. 5). The rbCII959K and rbCII959A proteins had no activity at all (data not shown).

Confocal fluorescent microscopy with antibodies specific to rbCII showed that a significant fraction of the wild-type rbCII protein, used as a control, was localized to the plasma membrane in BHK6 cells (supplemental materials Fig. S1). However, some fraction appeared to remain in the cytoplasm. Almost all fractions of the rbCII959E, rbCII959S, and rbCII959A proteins were mainly present in the cytoplasm. By contrast, the rbCII959S protein was completely localized to the plasma membrane. These results indicate that the Gly-Ser substitution in rbCII results in marked attenuation of channel activity without altering the localization efficiency of the protein. The Gly-Glu, Gly-Lys, and Gly-Ala substitutions bring about a complete loss of channel activity, and this loss may be due to a decreased efficiency of protein transport to the plasma membrane.

DISCUSSION

Here we have shown the importance of the Gly residue present in the domain III S2–S3 linker of the yeast putative VGCC α1 subunit Cch1 and of the rat VGCC α1 subunit rbCII, a member of Ca1.2. Based upon a conventional transmembrane prediction (33), the domain III S2–S3 linker of VGCCs is composed of 13 amino acid residues and the Gly residue is the only one amino acid residue that is completely conserved in the linker (Fig. 2). This suggests that the Gly residue is indispensable for the function of VGCCs, and we have proven this suggestion.

Our study on the Cch1G1265E protein have indicated that Gly1265 is required for the function of Cch1, but probably not for the stability and subcellular localization of this protein because the amount of Cch1G1265E and the localization of Cch1G1265E-EGFP are essentially the same as those of the wild-type counterparts (Fig. 3, A and D). The reason for the importance of the Gly residue is that it has the smallest side chain, as revealed by systematic amino acid substitution experiments (Fig. 4).

As for rat rbCII, on the other hand, Gly959 may be important for not only function but also localization. The rbCII959E, rbCII959S, rbCII959K, and rbCII959A proteins have no or very little Ba2+ current, as revealed by patch clamp analysis. Indirect fluorescent immunomicroscopy has shown that, among these mutant proteins, the majority of the rbCII959E, rbCII959K, and rbCII959A proteins seem to be localized in the cytoplasm, instead of localization to the plasma membrane (supplemental materials Fig. S1). In addition, many of even the wild-type rbCII protein appears to remain in the cytoplasm. We speculate
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FIGURE 4. Effect of the substitution of various amino acids for Gly^{2265} on Cch1 function. Cells of the cch1Δ mutant expressing a Cch1 protein having a mutation in Gly^{2265} from the self-promoter or TDH3 promoter were grown in SD.Ca100 medium, exposed to α-factor, and examined for viability and Ca^{2+} accumulation. A, the viability of cells was determined 8 h after exposure to α-factor. Open and closed bars represent the viability of cells expressing a mutant Cch1 protein produced from the self-promoter and the TDH3 promoter, respectively. Mean ± S.D. from at least three independent experiments. Plasmids used: vector, pBC111; CCH1 (G), pBCS-CCH1H; single letters from Glu to Lys represent the substituted amino acids at Gly^{2265}. Amino acid abbreviation: G, Gly; E, Glu; A, Ala; S, Ser; D, Asp; N, Asn; T, Thr; V, Val; Q, Glu; L, Leu; K, Lys. B, replot of the viability data shown in A as a function of the volume of amino acid residues (38). The data based on the self-promoter are shown. C, replot of the viability data shown in A as a function of the hydrophobicity of amino acid residues (39). The data based on the self-promoter are shown. D, Ca^{2+} accumulation was determined 2 h after the cells were exposed to α-factor. Open and closed bars represent the data obtained based on the self-promoter and the TDH3 promoter, respectively. Mean ± S.D. from at least three independent experiments. Plasmids used and amino acid abbreviations were the same as those in A. E, replot of the Ca^{2+} accumulation data shown in D as a function of the volume of amino acid residues. The data based on the self-promoter are shown. F, replot of the Ca^{2+} accumulation data shown in D as a function of the hydrophobicity of amino acid residues. The data based on the self-promoter are shown.

FIGURE 5. Effects of substitution of various amino acids for Gly^{959} on the Ca^{2+} channel activity of rbCII. Whole cell recordings of Ba^{2+} currents through the L-type Ca^{2+} channel rbCII and its mutant proteins expressed in BHK6 cells were shown. A, wild-type rbCII; B, the rbCIIG959E protein; C, the rbCIIG959S protein. The dysfunction of G959E could be due to the impairment of plasma membrane targeting (see supplemental materials Fig. S1). G959S showed markedly small Ba^{2+} currents, although it was localized to the plasma membrane (see supplemental Fig. S1). D, current-voltage relationships (I-V curve) of wild-type and mutant rbCIIIs (G959E and G959S).

that overexpression of rbCII mRNA driven by the strong CMV promoter might have resulted in the mislocalization of these proteins because of excess in the amount of the α_{1} subunit over that of the β and α_{2}/δ subunits. It is known that the correct targeting of the α_{1} subunit to the plasma membrane requires the β subunit (23). By contrast, rbCIIG959S seems to be efficiently transported to the plasma membrane. Taken together, it is likely that Gly^{959} is necessary for the function and localization of rbCII.

From a structural viewpoint of voltage-gated ion channels, we speculate that the Gly residue has a role for the formation of a voltage-sensing structure composed of the S1–S4 segments. Among six transmembrane segments, the S1–S4 segments are considered to function as the voltage sensor of structurally related, voltage-gated Ca^{2+}, K^{+}, and Na^{+} channels (2). In the case of K^{+} channels whose structure is best elucidated, the four most extracellularly located basic residues of the S4 segment and the most intracellular acidic residue in the S2 segment are the major contributors to the gating charge movement that is coupled with the opening or closing of the pore of the channel (34, 35). In addition, according to the helix-packing model, the interaction of conserved negative charges in S2 and S3 with the conserved positive charges in S4 makes S2 and S3 line one side of the gating canal (36, 37). Thus, it is likely that the structure of the S2–S3 linker is also important for channel gating and the replacement of the Gly residue with any larger amino acid perturbs the spatial arrangement of S2 and S3. This perturbation may also affect the localization efficiency of VGCC α_{1} subunits, as seen in rbCII.

Finally, we would like to point out that the Gly residue of the S2–S3 linker is present not only in domain III but also in domains I, II, and IV with a slight exception (data not
shown). Therefore, the Gly residue should have an essential function in all of the four domains.

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