A TRP homolog in *Saccharomyces cerevisiae* forms an intracellular Ca^{2+} -permeable channel in the yeast vacuolar membrane

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The molecular identification of ion channels in internal membranes has made scant progress compared with the study of plasma membrane ion channels. We investigated a prominent voltage-dependent, cation-selective, and calcium-activated vacuolar ion conductance of 320 pS (yeast vacuolar conductance, YVC1) in *Saccharomyces cerevisiae*. Here we report on a gene, the deduced product of which possesses significant homology to the ion channel of the transient receptor potential (TRP) family. By using a combination of gene deletion and re-expression with direct patch clamping of the yeast vacuolar membrane, we show that this yeast TRP-like gene is necessary for the YVC1 conductance. In physiological conditions, tens of micromolar cytoplasmic Ca^{2+} activates the YVC1 current carried by cations including Ca^{2+} across the vacuolar membrane. Immunodetection of a tagged YVC1 gene product indicates that YVC1 is primarily localized in the vacuole and not other intracellular membranes. Thus we have identified the YVC1 vacuolar/lysosomal cation-channel gene. This report has implications for the function of TRP channels in other organisms and the possible molecular identification of vacuolar/lysosomal ion channels in other eukaryotes.

Ion channels are universal molecular entities found from prokaryotes to higher eukaryotes (1). Unitary conductances have now been recorded by patch clamp from *Dictyostelium* (slime mold), *Paramecium* (a ciliate), *Neurospora* (bread mold), *Uromyces* (a parasitic bean rust fungus), budding and fission yeasts, *Escherichia* and *Streptomyces* (Gram-negative bacteria), *Bacillus* (a Gram-positive bacterium), and *Haloferax* (an archaeon) (2). Microbial genomes have also revealed many putative ion-channel homologs, although only a few have been correlated with the conductances recorded.

Within the yeast genome, several sequences have homology to known channels (3). They include TOK1 (a two-pore K^+ channel) (4), CCH1 (a Ca^{2+} -channel homolog) (5), CLC1 (6) (a Cl^- -channel homolog) and MID1 (a mating-related channel, see ref. 7). In parallel, patch-clamp surveys of the plasma membrane of yeast cell reveal a 30-pS outwardly rectifying K^+ conductance, which has been identified as TOK1 (4), and a 40-S mechanosensitive conductance, the identity of which is unknown (8). Patch clamp has also been applied to yeast vacuolar membranes. Several groups have reported a >100-S cation conductance in the vacuolar membrane (9–11). Here we show this conductance to be dependent on a TRP-like gene, which we name YVC1. The identification of the YVC1 is of particular interest. Although cation conductances have been recorded from organelles such as the endoplasmic reticulum of cerebral cortex (12) and the vacuoles of plants (13) and yeast (9–11), the molecular identity of these activities has remained elusive. To date, apart from ryanodine and inositol trisphosphate receptors, few genes encoding intracellular cation conductances have been identified. As a result there is a paucity of information concerning the role of intracellular ion channels in cell physiology.

Materials and Methods

Yeast Proteome Database Search, National Center for Biotechnology Information BLAST Search, Protein Analysis Programs. Analysis of the yeast genome was performed using the Yeast Proteome Data-

base at Proteome (www.proteome.com). Putative ORFs with transmembrane (TM) domains of unknown function were BLAST searched against the National Center for Biotechnology Information nonredundant database (www.ncbi.nlm.nih.gov). The YVC1 gene was analyzed by using the DNASTAR program PROTEAN (www.dnastar.com) using a window of 12 amino acids. The TRP family of genes including YVC1 were aligned and compared with the DNASTAR MEGALIGN program by using a PAM250 residue weight table.

Yeast Strains. YVC1: *S. cerevisiae* parental strain BY4742 (*his3D1 leu2D0 lys2D0 ura3D0 MAT α*). YVC1 Δ : (YVC1:km): BY4742 containing a complete chromosomal replacement of the entire YVC1 gene (YOR087/88w) with a kanamycin-resistance gene. YVC1 Δ [pYVC1]: YVC1 Δ containing the expression plasmid (PCM190^{Zeo})-borne copy of the YVC1 gene under the control of a repressible promoter (doxycycline repressible). YVC1 Δ [pEmpty]: YVC1 Δ containing an empty plasmid (PCM190^{Zeo}). YVC1-HA: Genomic copy of YVC1 in BY4742 replaced with a hemagglutinin (HA)-tagged copy of YVC1.

Gene Deletion and Vector Construction. Yeast genomic deletions were constructed in strain BY4742 by using a kanamycin-resistance cassette, and the entire deletion of YOR087/88w was confirmed by PCR analysis as described (14). The YVC1 gene was amplified from yeast genomic DNA of strain BY4742 by PCR and cloned into PUC18 for sequencing by standard methods. Yeast expression vector PCM190 (15), which contains a doxycycline-repressible promoter, was obtained from the American Type Culture Collection (www.atcc.org) and a Zeocin-resistance gene (Invitrogen) was inserted into the vector to allow selection with Zeocin (PCM190^{Zeo}). All yeast protocols and media were as described (16).

Electrophysiological Methods. As diagrammed in Fig. 3a, actively growing yeast cells were treated with wall-digesting enzymes, and spheroplasts were obtained by further incubating with either 10 mM glucose- or galactose-supplemented saline for 24 h. Vacuoles, released from the spheroplasts by lowering the bath osmolarity, are optically distinct and can form tight seals instantly with the pipet tip upon a gentle suction. Whole-vacuole mode can be reached by applying a few pulse shocks (0.5 V, 30 ms). During spheroplasting and patch-clamp recording, all solutions were supplemented with 2 mM DTT unless stated otherwise. These protocols, as well as the compositions of

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Abbreviations: YVC, yeast vacuolar conductance; TRP, transient receptor potential; HA, hemagglutinin; GFP, green fluorescent protein; ER, endoplasmic reticulum; TM, transmembrane.

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Fig. 1. Deduced amino acid sequence of the protein encoded by the *YVC1* gene and amino acid alignment with related sequences. (a) Deduced amino acid sequence of the *YVC1* gene; predicted TM domains 1–6 are underlined. The C terminus contains a putative calcium binding bowl (18) (DDDD motif residues 572–575). (b) Alignment between the predicted sixth TM region of *YVC1* and the predicted sixth TM regions of other TRPs: *Candida* TRP, a homolog in *C. albicans*; mTRP2 and mTRP4, mouse homologs (19, 20); OSM9, a homolog in *Caenorhabditis elegans* (21); dTRP, the *Drosophila* TRP (22).

various solutions, were as detailed (10, 11), with minor adjustments depending on the conditions of the cells. Recording conditions and the pipet or bath solutions are stated in figure legends. Membrane currents were recorded using an EPC7 patch-clamp amplifier (List Medical Systems) at room temperature. Signals were filtered at 1 kHz before digitized and analyzed with PCLAMP 6.0 software (Axon Instruments, Foster City, CA). Single-channel slope conductance was determined by a linear regression method from *I/V* curves.

Immunological Detection of the YVC1 Gene Product. The genomic copy of *YVC1* in BY4742 was replaced with an HA-tagged *YVC1* copy by homologous recombination (16). Patch clamp recording of BY4742 *YVC1*-HA indicated that the *YVC1* conductance was still intact (data not shown). Subcellular fractions were prepared from BY4742 *YVC1*-HA cells by using Accudenz density gradient centrifugation (Accurate Chemical and Scientific Corp.) as described (17). Eleven fractions were collected in total and separated by SDS/PAGE on quadruplicate gels. After Western blotting, the blots were probed with antibodies to an HA tag (Boehringer-Mannheim 12CA5), ALP1 (Molecular Probes, vacuole marker), VPS10 (Molecular Probes, golgi marker), and DPM1 [Molecular Probes, endoplasmic reticulum (ER) marker]. A horseradish peroxidase-labeled anti-mouse IgG antibody (Jackson Immunochemicals) with an ECL detection kit (Amersham Pharmacia) was used to detect the fractionated proteins.

Results

The yeast vacuolar membrane has a >100-pS cation conductance (9–11). To identify the gene encoding this conductance from the genome, 586 putative ORFs of unknown function with 3–12 predicted TM domains from the Yeast Proteome Database were BLAST searched against the National Center for Biotechnology Information database of protein sequences. Several ion-channel

homologs were observed in addition to those reported (3). The deduced amino acid sequence of two putative ORFs, YOR087/YOR088w (Fig. 1a), which we found to be a single continuous ORF upon resequencing, were found to have significant homology with the recently discovered mouse TRP2 (19) (14.9% amino acid identity) and other TRPs (Fig. 1b) (20–22). Hydrophilicity (Fig. 2a) and domain prediction (data not shown) indicate that

Fig. 2. Predicted structure of the protein encoded by the *YVC1* gene. (a) Hydrophilicity plot of the predicted protein encoded by *YVC1*. Six potential TM domains are marked (1–6). (b) Model structure for the protein encoded by the *YVC1* gene; six TM domains (1–6) and a putative pore region (P) are labeled. A putative calcium binding bowl in the C terminus of the protein is also marked (18).

Fig. 3. Absence and restoration of the yeast vacuolar conductance are correlated with the deletion and expression of the *YVC1* gene. (a) Diagrammatic representation of the method of vacuole preparation and patch-clamping. (b) Whole-vacuole macroscopic currents upon applying a voltage ramp from +70 to -70 mV (bath voltage, cytoplasmic side) from each of the three yeast strains as marked. (c) Sample traces from whole vacuoles held at +10 mV. The low open probability at this voltage allows a clear resolution of the unitary conductances as marked from closed (C), to open (O¹, O², O³). Bath solution: 150 mM KCl/100 μ M CaCl₂; pipet solution: 180 mM KCl/10 μ M CaCl₂; both also had 5 mM MgCl₂, 5 mM Hepes, and 2 mM DTT (pH 7.2).

YOR087/88 encodes a protein that contains six TM domains with long N and C termini similar to the topology predicted for some members of the TRP family (23–25) (Fig. 2b). The most significant homology to other TRP channels is found in the predicted sixth TM domain (Fig. 1b), which forms part of the ion conduction pathway and is intimately associated with deactivation gating in cation channels. The C-terminal portion contains a DDDD motif that may be involved in Ca²⁺ regulation similar to the Ca²⁺-binding bowl in Big K⁺ Ca²⁺-activated channels (18). We refer to this TRP gene as the yeast vacuolar channel (*YVC1*).

We used chromosomal deletion and gene re-expression in trans coupled with direct patch-clamp analysis of the vacuolar membranes to establish the role of *YVC1* in the 300-S conductance. In a near symmetric K⁺ solution, hyperpolarization (i.e., cytoplasmic-side negative) of wild-type vacuoles activated a prominent inward current from the vacuole into the cytoplasmic side as has been reported (9–11). This inwardly rectifying channel has an open probability (P_o) peaking at about -80 mV (cytoplasm negative) and falling to near zero at positive voltages (Fig. 3b and c; *YVC1*). In our recording conditions, this channel has a single-channel slope conductance of $\approx$ 320 pS. Whole-vacuole currents, combined with single-channel recording (data not shown), indicate a minimum of 100 channels per vacuole. This conductance has never been observed from the plasma membrane of yeast ($n > 500$; data not shown). This current is entirely missing in every one of over 50 vacuoles examined from the *YVC1*-deleted strain (Fig. 3b and c; *YVC1* Δ). Re-expression of *YVC1* in the *YVC1* Δ strain from a plasmid (p*YVC1*) with a repressible promoter clearly restored this current in the absence of the repressor ($n > 50$) (Fig. 3b and c). No increase in channel numbers was observed in the *YVC1* over-expressing strain compared with the wild-type strain. In the presence of the repressor,

the conductance was never observed ($n > 20$; data not shown). In identical conditions, the current was absent in the knockout background containing an empty plasmid in the presence or absence of the repressor (data not shown). No other conductances were observed in the vacuolar membrane under the conditions surveyed in either the *YVC1* or *YVC1* Δ strain. In both strains, the mechanosensitive conductance and the TOK1 K⁺ conductance in the plasma membrane appeared normal. Thus, we conclude that the *YVC1* gene is necessary for the yeast vacuolar conductance.

In all strains surveyed, the growth of the cells and the appearance of the cells and vacuoles were comparable by phase-contrast microscopy. No apparent phenotype has been detected for the *YVC1* Δ strain. Yeast functional genomic projects, as yet, have indicated no apparent difference between the *YVC1* Δ and the *YVC1* strain for a number of growth conditions (www.stanford.edu/Saccharomyces/). We further tested and found that the *YVC1* haploid and diploid knockout strains are without apparent defects in vegetative growth, sporulation, mating, or pseudohyphal growth (data not shown). Thus, either *YVC1* is functionally redundant (nonhomologous redundant mechanisms may exist) or it is used under certain acute biological stresses not yet simulated in the laboratory.

Although we have clearly shown *YVC1* to be necessary for the YVC conductance, we have not proven that this gene alone is sufficient in encoding the pore-forming subunit of this channel. This is due to the lack of a system to heterologously express intracellular ion channels. When we expressed *YVC1* in *Xenopus* oocytes, no additional conductances were observed on the oocytes' plasma membranes (data not shown).

It has been shown that inositol trisphosphate (IP₃) releases vacuolar Ca²⁺ through an as yet unknown channel (26). Additionally, mTRP3 is activated by IP₃ by direct interaction with the IP₃ receptor (27). We tested the effects of IP₃ on the modulation of *YVC1* under a number of patch-clamp conditions, but found no apparent evidence of modulation (data not shown). Some TRP channel members are modulated by polyunsaturated fatty acids (arachidonic acid and linolenic acid) (28). We also tested the ability of these substances to modulate *YVC1*, but no clear evidence of modulation was seen (data not shown). Additionally, we also tested the ability of cAMP to modulate *YVC1*, but no evidence of modulation was found (data not shown).

In *Candida albicans*, a vacuolar voltage-dependent Ca²⁺-activated Ca²⁺-release mechanism was demonstrated by fluorescence quench ion flux assays (29). Given the 45% amino acid homology between the *YVC1* of *S. cerevisiae* and the predicted *C. albicans* TRP (Fig. 1b), it is possible that this Ca²⁺-release mechanism is encoded by the *Candida* TRP gene. Similar experiments repeated in *S. cerevisiae* did not reveal a vacuolar Ca²⁺-activated Ca²⁺-release mechanism (data not shown). Vacuolar Ca²⁺ release during homotypic vacuole fusion has also been reported (30); however, deleting *YVC1* did not abolish vacuole fusion (A. Mayer and C.P., unpublished results).

Functions of ion channels are often inferred from their gating principles and ion selectivity. We have re-examined the wild-type *YVC1* channel activities and confirmed Ca²⁺ activation. Activation clearly occurs at millimolar free Ca²⁺ at the cytoplasmic side (Fig. 4a, without DTT). Reducing agents such as DTT and 2-mercaptoethanol enable the channel to be activated at tens of micromolar Ca²⁺ (data similar to Fig. 4a, not shown). The requirement for gating is Ca²⁺ specific; Mg²⁺, Ba²⁺, or Mn²⁺ does not activate even in high concentrations (data not shown). This channel is apparently permeable to a variety of cations (9–11), although Ca²⁺ permeation has not been unambiguously demonstrated. Because of the physiological importance of Ca²⁺, we have also re-examined the permanent ions and discovered that *YVC1* can pass a cation current between the pipet and the bath when Ca²⁺ is the sole cation on both sides (Fig. 4b). Thus,

Fig. 4. Ca^{2+} -related properties of the wild-type YVC1 current. (a) Whole-vacuole recording at -30 mV (cytoplasm negative) showing the absence or presence of transmembrane current (traces) at $0.1 \mu M$ or $1 mM$ Ca^{2+} , adjusted with EGTA, in the bath (cytoplasmic side). Bath: $150 mM$ KCl; pipet: $180 mM$ KCl; both also had $5 mM$ $MgCl_2$ and $5 mM$ Hepes, pH 7.2. Amplitude distribution histogram displays data from a 20-s recording, ≈ 0.5 s of which are shown in the second trace. The whole vacuolar YVC1 mean open probability is 10. (b) YVC1 current can be carried solely by Ca^{2+} . Whole-vacuole macroscopic currents with symmetric Ca^{2+} as the only permeant ion. Polarizations or depolarizations were applied to the patch as indicated. Channel openings are marked O^1 and O^2 . Symmetric solution: $120 mM$ $CaCl_2/2 mM$ DTT/ $5 mM$ Hepes (pH 7.2).

YVC1 can clearly serve as a conduit for the release of Ca^{2+} stored in the vacuole, when the cytoplasmic side first received a stimulatory amount of Ca^{2+} .

We investigated whether YVC1 is located exclusively in the vacuole, or in other intracellular membranes as well. The genome copy of YVC1 in BY4742 was replaced with an HA-tagged YVC1 copy. Yeast cells were collected and subcellular fractions were prepared by density gradient centrifugation (as described in *Materials and Methods*). Fractions were collected and separated on SDS/PAGE gels, followed by Western blot transfer. The four blots were probed with an anti-HA tag antibody and antibodies to markers for the vacuole (ALP1), Golgi (VPS10), and ER (DPM1). The results indicate that the major localization of the YVC1 gene product is in the vacuole. No HA-tagged YVC1 was detected in the ER or golgi (Fig. 5). Additionally, the genomic copy of YVC1 was tagged with green fluorescent protein (GFP) at the C terminus; however, no fluorescence was observed in the yeast. When the tagged GFP-YVC1 construct was over-expressed under the control of a Gal promoter (pGal426), clear fluorescence was observed. However, this fluorescence did not appear in the vacuole, but in the ER (data

Fig. 5. Localization of the YVC1 gene product. Western blot and immunodetection of HA-tagged YVC1, ALP1 (vacuole marker), VPS10 (Golgi marker), and DPM1 (ER marker) from subcellular fractionations (SF 1–11) of an HA-tagged YVC1 yeast strain. Both unprocessed ALP (ProALP) and mature ALP (MaALP) are detected, but only MaALP is located in the vacuole. Molecular masses of the bands are given in kilodaltons.

not shown), although YVC1 was still apparent in the vacuole by patch clamp recording. Given the fact that over-expression of YVC1 does not significantly increase the number of functional channels in the vacuole as measured with whole vacuole patch recording, we conclude that over-expression of the YVC1 gene product causes a build-up of YVC1 in the ER with no apparent deleterious effect on yeast growth (data not shown).

Discussion

The product of the transient receptor potential (TRP) gene was originally discovered as part of the *Drosophila* phototransduction cascade. TRP contains hydrophobic segments that are homologous to the TM segments of voltage-dependent Ca^{2+} and Na^{+} channels (22–24). Recently, a family of TRP genes have been discovered in mammals (mTRP 1–7), and protein database searching reveals a large family of TRP-resembling genes in animals ranging from *Caenorhabditis elegans* to *Homo sapiens* (25). It is thought that mammalian TRP channels are involved in capacitative calcium entry or act as store-operated Ca^{2+} channels, however, the precise function and physiological role of these genes is still under investigation (25, 31, 32). More recently, a TRP-related channel in *Drosophila*, nompC, has been implicated in mechanotransduction (33). We have shown here that the YVC1 gene encoding a TRP homolog is necessary for a vacuolar conductance that is localized exclusively in the vacuole. Thus, TRP homologs appear to encode conductances in an even wider range of functions and cellular locations than previously thought. This report appears to be the first instance that members of a

cation-channel family function in both the plasma membrane and in an intracellular membrane.

Although *YVC1* apparently encodes the pore-forming subunit of this channel, the physiological function of this channel is not clear. The slow calcium-permeable calcium-induced conductances (SV) observed in plant vacuoles possess many similarities to the YVC1, although the voltage dependence of YVC1 is opposite to that of SV channels (13, 34). These channels have been proposed to act as calcium-activated calcium release channels (35, 36). In the case of YVC1, it is clear that Ca^{2+} , as the permeant ion, may flow either from the vacuole to the cytoplasm or vice versa (Fig. 4b). Because ion channels are passive devices through which ions pass along their electrochemical gradient, we feel it more likely that YVC1 acts as a Ca^{2+} -induced Ca^{2+} -release channel, given the predicted concentration of free Ca^{2+} in the vacuole at physiological pH (pH 5.6). Nonphysiologically high concentrations of Ca^{2+} (millimolar) are required to activate YVC1 in the absence of DTT (9–11). However, in the presence of DTT, physiological Ca^{2+} concentration (tens of micromolar) activates YVC1. The need for DTT in the recording solutions for physiological Ca^{2+} activation of YVC may be an indication that YVC1 is involved in responding to oxidative/reducing conditions in the yeast cell as suggested for plant vacuolar ion channels and animal ion channels (37). Alternatively, the DTT may simply

serve to mimic the constitutive redox environment inside the yeast cell.

Mucopolipidosis type IV (MLIV) is an autosomal recessive, neurodegenerative, lysosomal storage disorder characterized by psychomotor retardation and ophthalmological abnormalities (38). Recently the gene causing MLIV was mapped and found to contain a TRP channel homolog (39). Although the basic metabolic defect in MLIV has not been identified, it is known that fibroblasts from MLIV patient tissues are defective in lysosomal trafficking (40). It is possible that YVC1 is a functional homolog of MLIV.

To date, several ion channel activities have been recorded in yeast, including an outwardly rectifying voltage-gated potassium ion channel (TOK1) (4), YVC1 (9–11), and an as yet unidentified mechanosensitive plasma membrane ion channel (8). Microbial ion channels have contributed greatly to our structural understanding of this class of molecules (1). As yet, the study of the function of microbial ion channels has received scant attention. With the power of yeast genetics, it is now possible to investigate the function of this vacuolar conductance in the physiology of yeast and its relationship to other TRP genes from other organisms.

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