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Overexpression and purification of the vanilloid receptor in yeast (*Saccharomyces cerevisiae*)

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Abstract

The vanilloid receptor type 1 (VR1) is a novel membrane receptor activated by heat or chemical ligands conveying information about chemosensitive and thermosensitive pain. We have overexpressed and purified wild type VR1 (wtVR1) as well as several mutant forms using the yeast strain *Saccharomyces cerevisiae* with the goal of obtaining sufficient protein for structural studies. To facilitate the rapid assaying of protein production and purification we used PCR to construct mutant VR1-green fluorescent protein fusion genes. All recombinant inserts were engineered with 12 HIS tags on the C-terminus for metal affinity column purification. The yield of purified protein from 16 L fermentation was about 1 mg following a single-step purification procedure. By taking advantage of the calcium permeability of VR1 we measured changes in $[Ca^{2+}]_i$ in capsaicin-stimulated fura-2 loaded yeast cells expressing VR1.

Keywords: VR1; Vanilloid receptor; Overexpression; Fusion proteins; Protein purification; Fura2

Vanilloid receptor type 1 (VR1) is an integral membrane protein that is activated by thermal energy and the chemical ligand capsaicin, the pungent extract from hot chili peppers. The gene was cloned from a dorsal root ganglion library [3]. The receptor is expressed in both the CNS and PNS. Using recombinant technology, VR1 can be expressed in oocytes or mammalian cell lines. Under these conditions, the receptor responds to capsaicin or an increase in temperature with a threshold of near 40 °C [3,15], a temperature that activates heat nociceptors in cats and monkeys [1,2,16,17]. Direct activation of VR1 by heat is supported by single channel analysis in excised membrane patches expressing the receptor [15]. In these studies, single channel activity was evoked by either capsaicin or thermal stimuli and the current elicited by each stimulus was blocked by the competitive capsaicin antagonist capsazepine. Predicted membrane topology based on hydrophobicity plots of the 838 amino acid protein features six transmembrane domains, several PKA and PKC consensus phosphorylation sequences, and three ankyrin repeats on the intracellular N-terminal domain [3]. VR1 is a homologous member of the transient receptor potential (TRP) family of store-operated Ca^{2+} channels [3,19,20]. Both the N- and C-termini are intracellular. When activated, VR1 forms a transmembrane nonspecific cation channel with high permeability to Ca^{2+} (permeability sequences $Ca^{2+} > Mg^{2+} > Na^{+} \approx K^{+} \approx Cs^{+}$). A reduction in pH dramatically lowers the threshold for VR1 activation, such that at pH 6.3, substantial currents are evoked at temperatures as low as 35 °C, a temperature at which the channel is normally closed [15]. Acidification also increases the potency of both capsaicin and heat by increasing channel open probability [6,15]. In fact, acidic bath solutions evoke ionic currents when applied to outside-out, but not inside-out, membrane patches excised from VR1-expressing HEK293 cells, suggesting that protons interact with an extracellular site(s) on the channel complex [15]. Candidate sites for such interactions include several acidic amino acids located within putative extracellular loops of VR1. Site-directed mutagenesis has pointed to two glutamate residues, one at position 600 and another at position 648 [6]. Introduction of neutral or positive residues at position 600 located between the putative fifth transmembrane domain

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and pore loop increases the responses of VR1-expressing cells to capsaicin or heat. For example, E600Q mutant channels show a >10-fold increase in sensitivity to capsaicin and E600K mutants show a dramatic decrease in thermal activation threshold (30–32 °C). These studies support the potential involvement of this region of the protein in the structural changes underlying molecular gating.

Structural studies of membrane proteins have been notoriously difficult. Due to their hydrophobicity they can only be removed from membranes and their solubility maintained in the presence of detergents. In addition, many membrane proteins are too large for structural determination by NMR techniques. The atomic structures of a few membrane proteins have been reported from an analysis of the X-ray diffraction pattern obtained from crystals [4,8,10,11]. However, crystallization trials require large amounts of pure protein to determine the conditions for crystal formation. Several approaches for overexpressing mammalian proteins in mammalian or insect cell lines, bacteria or yeast have been developed with limited success [12,13,18]. In this paper, we describe a method for overexpression and purification of wild type VR1 and several mutant forms with the goal of obtaining sufficient purified protein for structural studies.

Experimental procedures

Construction of expression vectors. The original rat VR1 clone in the pcDNA3 vector was a generous gift from Dr. D. Julius (University of California San Francisco). The pEGFP-N1 and pcDNA3.1 vectors were purchased from Clontech (Palo Alto, CA, USA). To simplify protein detection during the overexpression and purification trials, we constructed a colored fusion protein of VR1 by adding the cDNA sequence for enhanced green fluorescent protein (eGFP) to the C-terminus. This allowed us to screen the product by irradiation with UV light to observe the green fluorescence during a number of steps in the protocols for overexpression and purification trials. The original VR1 construct was modified by PCR to include two restriction enzyme sites, *Eco*RI at the 5′ end and *Kpn*I at the 3′ end. By enzymatic digestion with *Eco*RI/*Kpn*I and *Kpn*I/*Not*I we removed VR1 from the pcDNA3 multiple cloning site and eGFP from its vector. The two fragments were ligated and subcloned into the *Eco*RI/*Not*I site of pcDNA3.1. This construct was named VR1/eGFP.

A deletion mutant of VR1 was made by removing the N-terminus of VR1 up to Q423. This deletion mutant was called $VR1^{(-)}$/eGFP. By engineering two restriction enzyme sites *Eco*RI at the deletion site and *Xba*I at the 3′ end, $VR1^{(-)}$/eGFP was subcloned into *Eco*RI/*Xba*I sites of pcDNA3.1.

Cloning into yeast expression plasmid. The yeast expression plasmid YEpHIS is a leucine selectable 2μ episomal yeast expression shuttle vector with strong constitutive promoter PMA1 and was a generous gift from Dr. M. Al-Shawi (University of Virginia). Plasmid YEpHIS has insertion sites *Spe*I at the 5′ end and *Mlu*I at the 3′ end. By PCR we added the *Spe*I site at the 5′ end and *Mlu*I site and 12 histidine codons (histidine tag) at the 3′ end. wtVR1, VR1/eGFP, and $VR1^{(-)}$/eGFP were removed from pcDNA3.1 by restriction enzyme digest and cloned into the *Spe*I/*Mlu*I sites of YEpHIS. Constructs were verified by DNA sequencing. All enzymes were purchased from Promega (Madison, WI, USA), New England Biolabs (Beverly, MA, USA), or Invitrogen (Carlsbad, CA, USA).

Yeast strain, transformation, and expression. The protease deficient *S. cerevisiae* yeast strain BJ5457 (ATCC) used for overexpression was grown in YPD broth (Sigma, St. Louis, MO, USA) prior to transformation. Yeast cells were transformed by lithium/cesium acetate using the alkali-cation yeast transformation kit (QBIOgene, Carlsbad, CA, USA) and plated on SD agar lacking leucine (SDA-LEU, QBIOgene, Carlsbad, CA, USA). Transformants were transferred into 15 ml Falcon tubes (Becton–Dickinson, Franklin Lakes, NJ, USA) with SD media lacking leucine (SD-LEU, QBIOgene, Carlsbad, CA, USA) and with varying concentrations of glycerol. Precultures were inoculated at 0.2 OD_{600} units into 1-L Tunair Shaker Flasks (Shelton Scientific, Shelton, CT, USA) in SD-LEU and grown at 25 °C with constant agitation (200 rpm). These small-scale tests were used to develop the parameters for maximum growth and purification methods for the different constructs. For large-scale overexpression, yeast cells were grown in a 16-L fermenter (UTMB Protein Laboratory, Galveston, TX, USA).

Isolation of plasma membranes and purification. All procedures were performed at 4 °C. Yeast cultures were harvested at 1.0–1.5 OD_{600} units by centrifugation at 3000*g* for 10 min. Cells were resuspended in 5 volumes of homogenization buffer (50 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM PMSF) in the presence of yeast protease inhibitor cocktail (Sigma, St. Louis, MO, USA) and lysed in a microfluidizer (M-110EH, Microfluidics, Newton, MA, USA) at 20,000 psi. The lysates were centrifuged at 3000*g* for 5 min. The supernatant was removed and centrifuged at 14,000*g* for 20 min. Supernatant fractions were then centrifuged at 200,000*g* for 1 h. Pellets were resuspended in 50 ml of membrane washing buffer (10 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 1 M NaCl, 1 mM β-mercaptoethanol, and 1 mM PMSF), homogenized, and centrifuged at 200,000*g* for 1 h. The pellet consisting of crude membranes was resuspended in storage buffer (10 mM Tris–HCl, pH 7.5, 0.3 M sucrose, 1 mM β-mercaptoethanol, and 1 mM PMSF) at a concentration of 5 mg/ml, divided into aliquots, and stored at −80 °C.

Crude membranes were solubilized, by gentle rotation, overnight at 4 °C at a protein concentration of 2 mg/ml in solubilization buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF, and different detergents [Triton X-100, SDS, *n*-octyl-β-D-glucoside (OG), *n*-dodecyl-β-D-maltoside (DDM), egg L-α-lysophosphatidylcholine (LPC), and deoxycholate (CH)]. Suspensions were centrifuged at 100,000*g* for 1 h and supernatants were batch loaded, by gentle rotation, overnight at 4 °C onto a cobalt-affinity resin (TALON Metal Affinity Resin, Clontech, Palo Alto, CA, USA) pre-equilibrated with solubilization buffer. The resin was loaded into a Kontes Flex-Column (Fischer Scientific, Houston, TX, USA) by gravity and washed with an imidazole gradient wash buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 10–60 mM imidazole in 10 mM steps of 5 volumes of wash buffer to 1 volume of resin, 1 mM β-mercaptoethanol, 1 mM PMSF, and 0.01% *n*-octyl-β-D-glucoside). Purified proteins were eluted with elution buffer (wash buffer but with 300 mM imidazole and 0.05% *n*-octyl-β-D-glucoside). Proteins were concentrated and dialyzed overnight against 50 mM Na_2HPO_4, 300 mM NaCl, and 0.05% *n*-octyl-β-D-glucoside at pH 7.5. Total protein concentrations were measured using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). All general chemicals were purchased from Sigma (St. Louis, MO, USA) and detergents were purchased from Anatrace (Maumee, OH, USA).

Electrophoresis, Western blotting. Crude membranes or purified proteins were subjected to 10% SDS–polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) electrophoresis and transferred on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The presence of wtVR1, VR1/eGFP, and $VR1^{(-)}$/eGFP was assayed by using a C-terminal VR1 antibody (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and enhanced by chemiluminescence (Pierce, Rockford, IL, USA). Polyacrylamide gels were

Coomassie blue-stained (Pierce, Rockford, IL, USA) to verify the presence of purified proteins.

Measurement of $[Ca^{2+}]_i$ in expressing yeast. Yeast were grown to an OD_{600} of 1.2–1.5 and loaded with fura-2 AM (100 μM) by incubation overnight at 4 °C. Cells were transferred into 4 ml cuvettes that contained a small stir bar. The cuvette was placed in the chamber of a SPEX Fluorolog Spectrofluorimeter Spectrometer (SPEX Industries, Edison, NJ, USA) fitted with a magnetic stirrer. The emission above 510 nm was measured during an excitation scan from 300 to 400 nm. Capsaicin 1–10 μM final concentration was added directly to the cuvette. In the presence of capsaicin, the decrease in fluorescence around 380 nm and the increase around 340 nm are indicative of an increase in $[Ca^{2+}]_i$.

Results

The physical map showing the yeast plasmid with a generic VR1 insert is shown in Fig. 1A. The only difference between plasmid constructs was the sequence of the VR1 insert in the multiple cloning sites between *Spe*I and *Mlu*I. *S. cerevisiae* has the advantage that the eukaryotic biogenesis and trafficking machinery are present and the plasmid is easily modified [9]. Fig. 1B is a Western blot of yeast membranes showing the expression of the 125 kDa VR1/eGFP (lane 1) and 78 kDa VR1$^{(-)}$/eGFP (lane 2) constructs. Each lane shows a double band, which is attributed to glycosylated forms of the protein [7,14].

Expression of membrane proteins in yeast can be enhanced by chemical chaperones. Glycerol has been shown to stabilize the conformation and enhance the expression level of some membrane proteins [5]. To establish the best conditions for overexpression, we investigated the effect of glycerol on each construct and our results are shown in the Western blots of Figs. 2A and B. Yeast were harvested at OD_{600} of 1.2 (24 h) grown in different concentrations of glycerol. The concentration of glycerol that produced the highest expression was 5% for both wtVR1 and VR1/eGFP (not shown) and 10% for VR1$^{(-)}$ and 15% for VR1$^{(-)}$/eGFP (not shown). Generally, for the smaller constructs more glycerol was necessary to obtain maximum protein expression. Fig. 2B shows the level of expression of wtVR1 and VR1$^{(-)}$ at different times. Maximum expression occurred at 24 h for all constructs and subsequently, yeast were harvested at this time.

Several detergents were screened to determine the most effective for protein extraction. Different concentrations and incubation times were tried to assay the

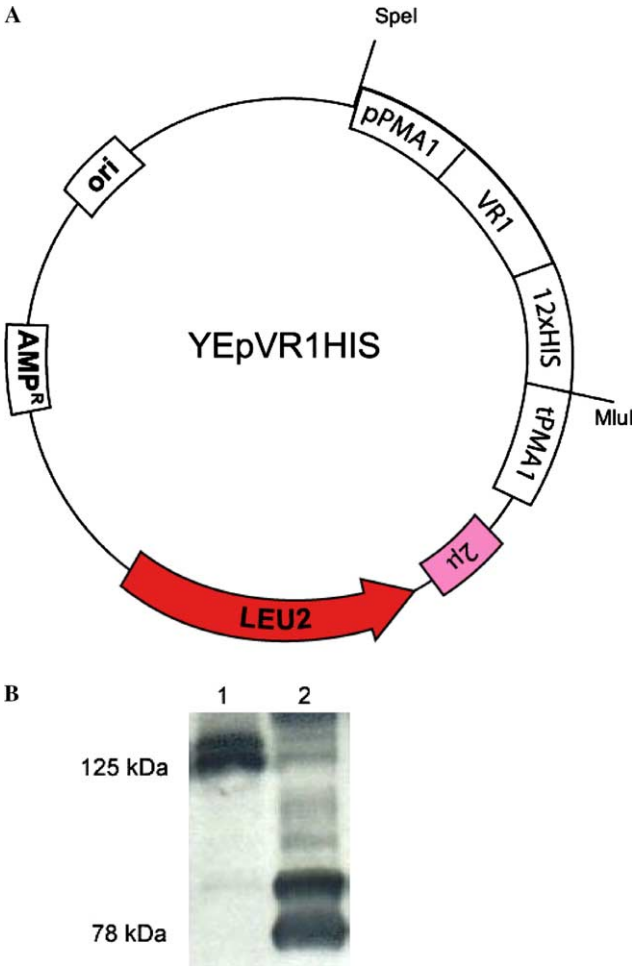

Fig. 1. (A) Yeast plasmid YEpVR1HIS. (B) Western blot of yeast membranes probed with anti-VR1. Lane 1, VR1/eGFP and lane 2, VR1$^{(-)}$/eGFP.

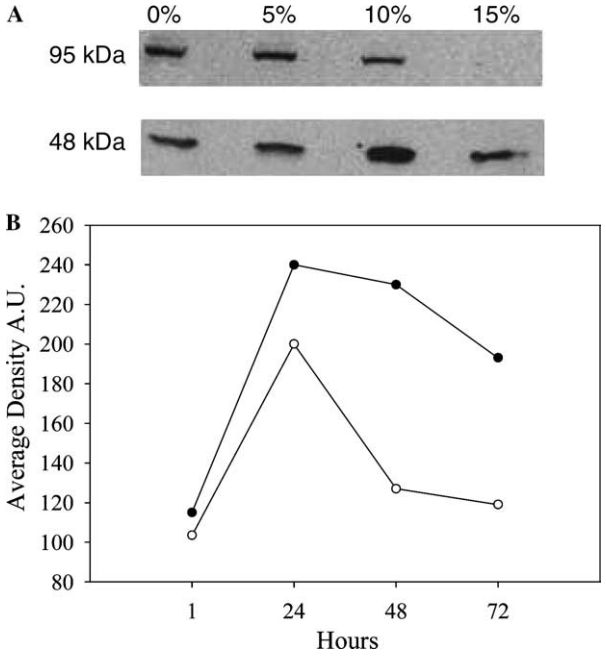

Fig. 2. (A) Western blot showing the effect of glycerol on the level of protein expression for wtVR1 (top row) and VR1$^{(-)}$ (bottom row). The best expression levels were obtained with 5% glycerol for wtVR1 and VR1/eGFP and 10% for VR1$^{(-)}$. This was increased to 15% for VR1$^{(-)}$/eGFP. (B) Growth of yeast as a function of time. Maximum growth occurred at 24 h. All cultures were harvested at this time. Filled circles, VR1$^{(-)}$; open circles, wtVR1.

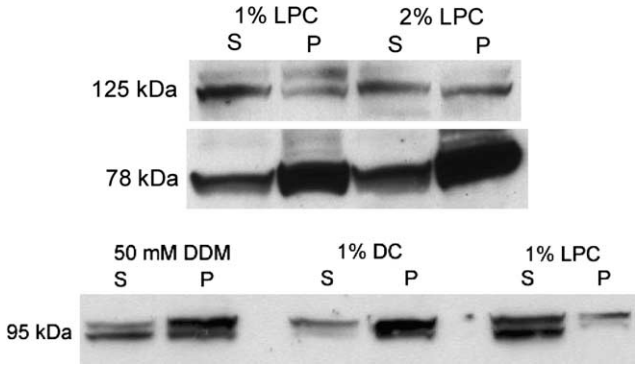

Fig. 3. Western blots showing the amount of protein extracted from yeast membranes using LPC for VR1/eGFP (top), VR1$^{(-)}$/eGFP (middle), and DDM, DC, and LPC for wtVR1 (bottom). Amount of total protein loaded/well was 60 μg. Supernatant (S), pellet (P). Solubilization 12 h at 4 °C with rotation.

importance of these two parameters. The detergents used include SDS, *n*-octyl-β-D-glucopyranoside (OG), Triton-X 100, sodium deoxycholate (DC), *n*-dodecyl-β-D-maltoside (DDM), and egg L-α-lysophosphatidylcholine (LPC). Although SDS was very good at extracting protein from yeast membranes, it would likely denature the protein and it was used only to compare the effectiveness of the other detergents. Good results were achieved with 1% egg L-α-lysophosphatidylcholine and *n*-dodecyl-β-D-maltoside (50 mM). However, a disadvantage of LPC is that it is difficult to remove during the purification steps. A Western blot showing the results of detergent solubilization on VR1/eGFP, VR1$^{(-)}$/eGFP, and wtVR1 constructs is shown in Fig. 3.

The fluorescence of eGFP was used to estimate the percent protein extracted from yeast membranes for the different detergents. Following the overnight rotation in detergent at 4 °C, the sample was centrifuged. The fluorescence in supernatant containing solubilized protein was compared with the total fluorescence measured in the resuspended pellet in supernatant. The ratio was calculated to give a percentage. The results for all of the detergents tested on VR1/eGFP are shown in Fig. 4. SDS extracted greater than 80% of the protein in yeast membranes. Egg L-α-lysophosphatidylcholine and n-dodecyl-β-D-maltoside extracted about 50% and 40%, respectively, and these detergents are unlikely to denature the protein.

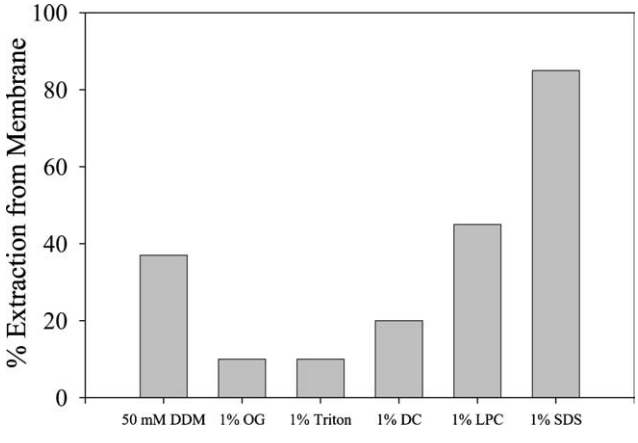

Fig. 4. The percent of VR1/eGFP extracted from yeast membranes for the different detergents measured from the amount of fluorescence. The fluorescence emission was measured above 525 nm at an excitation of 488 nm. LPC and DDM extract about 50% and 40% of the protein, respectively. Each trial was an overnight extraction with rotation carried out at 4 °C.

Protein that had been solubilized with either LPC or DDM was subjected to purification procedures by nickel affinity column of the HIS-tagged protein. Fig. 5A shows a Commassie blue stained gel of VR1/eGFP and VR1$^{(-)}$/eGFP following the purification procedure using 1% LPC for protein extraction. The arrows indicate the purified bands for VR1/eGFP (lane 5) and VR1$^{(-)}$/eGFP

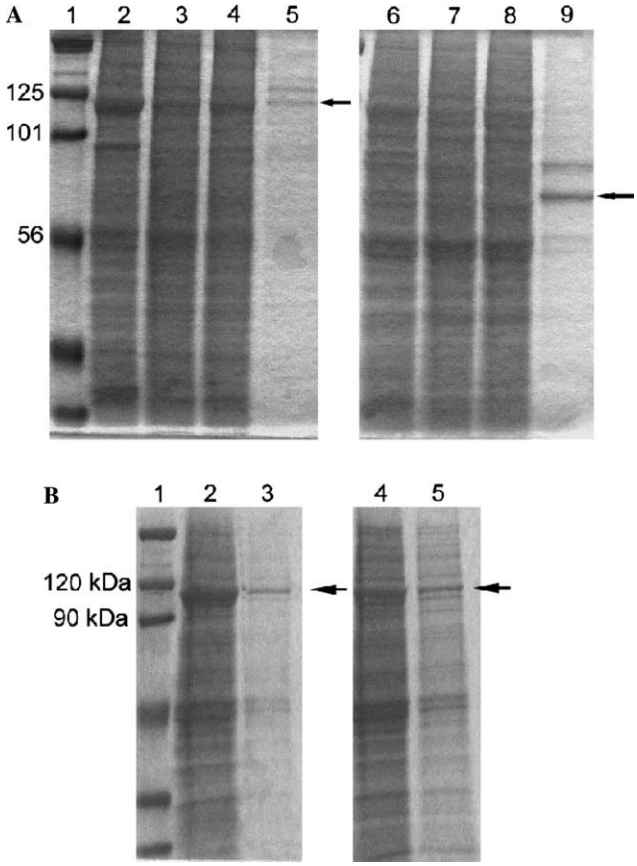

Fig. 5. (A) Coomassie-blue stained gel showing a one-step purification of VR1/eGFP (lanes 2–5) and VR1$^{(-)}$/eGFP (lanes 6–9). Lane 1, molecular markers; lanes 2 and 6, crude membranes; lanes 3 and 7, following 12 h at 4 °C in 1% LPC; lanes 4 and 8, unbound protein, and lanes 5 and 9, elution with 300 mM imidazole. Protein loaded in lane 5 was 6 ng and in lane 9, 12 ng. (B) Comparison of the one-step purification of wtVR1 solubilized 12 h in 50 mM DDM (lanes 2–3) and 1% LPC (lanes 4–5). Lane 1, molecular markers; lanes 2 and 4, crude membranes 60 μg total protein/lane; lane 3, 2.4 μg total protein/lane; and lane 5, 1.11 μg total protein/lane elution with 300 mM imidazole.

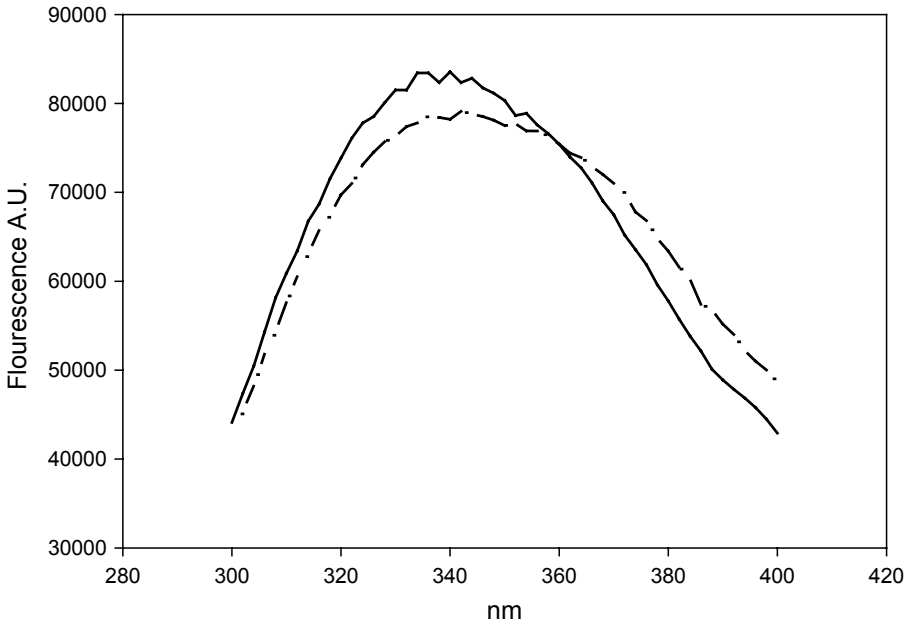

Fig. 6. Change in $[Ca^{2+}]_i$ in yeast expressing wtVR1 before (dashed line) and after (solid line) application of 1 μM capsaicin to the cuvette. The decrease in emission around 380 nm and the increase in emission around 340 nm are indicative of a rise in $[Ca^{2+}]_i$.

(lane 9). A similar experiment comparing purified protein extracted using DDM and LPC is shown in Fig. 5B for wtVR1. The Commassie blue stained gel shows that the first elution yields a prominent band near 95 kDa. It is clear that DDM yields a more pure fraction compared to LPC.

To assay for functional protein, the change in intracellular calcium was measured in fura-2 loaded yeast expressing wtVR1 before and after 1 μM capsaicin. Fig. 6 shows the fluorescence emission during an excitation scan from 300 to 400 nm. The decrease in fluorescence around 380 nm and the increase in fluorescence around 340 nm in the presence of capsaicin are indicative of an increase in intracellular calcium as would be expected for a functional calcium-permeable membrane channel like VR1.

Discussion

Small (<50 kDa) soluble proteins have been the focus of structural studies for a number of years because they are readily overexpressed and purified from bacterial systems which are easy to exploit, cost effective to produce, and for which a number of expression vectors are readily available. In addition, small proteins lend themselves to structural analysis using NMR methodology. Integral membrane mammalian proteins on the other hand are notoriously difficult to overexpress in conventional expression systems. The major stumbling blocks are developing the constructs that will overexpress the protein in a practical, cost effective, and easily manipulated system and establishing an expression system that will produce functional protein. Structural studies requiring a large amount of purified membrane proteins have often been restricted to those proteins that can be purified from natural sources that normally express sufficient quantity or for which orthologs are found in bacteria [4,8,11]. A prime example is rhodopsin that can be relatively easily purified from retinal rod outer segments [10]. Although a number of systems have been used to overexpress membrane proteins including insect cell lines, yeast, and bacteria the exact methodology for each protein must be established. In addition, it is necessary to assay the expressed protein for function. Often the expressed protein does not get to the membrane or it is incorrectly folded resulting in non-functional protein. The use of efficient heterologous expression systems as a source of abundant protein is therefore essential.

It is clear that the overexpression of an integral membrane mammalian protein is likely best done in as close to a native environment as possible. Mammalian proteins often require a specific lipid environment, post-translational modifications, and chaperones inherent to mammalian cells that may not be available in primitive eukaryote or in prokaryote expression systems. Hence, mammalian cell lines are the obvious choice for mammalian membrane proteins. Overexpression in a mammalian cell line however, if it succeeds at all, is cost and time ineffective. It is therefore important to try alternative expression systems to verify the expression of recombinant protein. In this study we demonstrate that VR1 and several mutant forms of the protein can be

overexpressed in yeast and purified from yeast membranes. VR1 constructs were cloned into a yeast plasmid with a strong PMA1 promoter. The level of overexpression is modulated by the chemical chaperone glycerol where the smaller construct requires lower concentrations of glycerol. This method is simple and cost effective.

The fusion protein constructs with eGFP facilitate rapid screening of the different steps required for overexpression, solubilization, and purification procedures using a spectrofluorimeter. Once the procedure has been established, constructs that do not contain eGFP can be used, however, for crystallization trials, the presence of eGFP has the added advantage that it allows for the positive identification of protein crystals as they will be green. We screened a number of detergents to determine those that would solubilize sufficient protein for purification. Both dodecyl-β-D-maltoside and egg L-α-lysophosphatidylcholine at either 1% or 2% were able to extract about 50% of the protein from yeast membrane fractions. Both detergents allow for purification on nickel columns. However, the eluate from samples solubilized with dodecyl-β-D-maltoside resulted in fewer unwanted proteins. This single step purification procedure yielded protein of >80% purity. In addition, LPC is difficult to remove a necessary step for crystallization trials or incorporation into liposomes. From 16 L fermentation, we estimate an amount of purified protein of about 1 mg.

In conclusion, we have demonstrated a simple and cost effective procedure for the overexpression and purification of VR1 and mutant forms of VR1. When expressed in yeast, VR1 is functional as demonstrated by calcium uptake in capsaicin stimulated yeast cultures. The method should provide sufficient material for structural studies.

Acknowledgments

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