Postprint: Establishment of In Vitro Research Methods for Store-Operated Calcium Channel Orai1

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Abstract

Objective: To establish an in vitro research method for the calcium channel Orai1. Methods: Utilizing liposome reconstitution technology, in vitro purified Orai1 protein was reconstituted into liposome membranes. Sucrose density gradient centrifugation was employed to assess reconstitution efficiency and the structural organization of Orai1 protein in the liposome membrane. Calcium ion release from within liposomes was detected using the calcium-sensitive dye Fura-2. Results: Liposomes were successfully prepared and GST-Orai1 fusion protein was purified in vitro. Sucrose density gradient centrifugation confirmed successful reconstitution of GST-Orai1 protein onto liposomes and demonstrated that Orai1 protein localized to the liposome membrane as multimers. Calcium ion release experiments verified that calcium ions were well encapsulated within the liposomes, enabling subsequent functional studies of the Orai1 calcium channel. Conclusion: A novel research method for Orai1 was established using liposome reconstitution technology, which enables more direct and effective investigation of its function and activation mechanism.

Full Text

Preamble

Establishment of an In Vitro Method to Study Store-Operated Calcium Channel Orai1

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Abstract

Objective: To establish an in vitro method for studying the calcium channel Orai1. **Methods:** Purified Orai1 protein was reconstituted into liposome membranes using liposome reconstitution technology. Sucrose density gradient centrifugation was employed to detect reconstitution efficiency and the structure of Orai1 protein in the liposome membrane, while the calcium dye Fura-2 was used to detect calcium ion release from within the liposomes. **Results:** Liposomes were successfully prepared and GST-Orai1 fusion protein was purified in vitro. Sucrose density gradient centrifugation demonstrated successful reconstitution of GST-Orai1 protein into liposomes, with Orai1 protein localized on the liposome membrane as multimers. Calcium release experiments confirmed that calcium ions were properly encapsulated within the liposomes, enabling subsequent functional studies of the Orai1 calcium channel. **Conclusion:** A novel method for studying Orai1 was established using liposome reconstitution technology, allowing for more direct and effective investigation of its function and activation mechanism.

Keywords: calcium channel; Orai1 protein; in vitro study

Introduction

Calcium ions serve as crucial intracellular signaling molecules that participate in nearly all cellular life activities, including gene transcription, cell secretion, energy metabolism, cell proliferation, differentiation, and apoptosis [1]. The foundation of calcium signaling lies in the concentration gradient between intracellular calcium ions and calcium stores or extracellular calcium. Alterations in intracellular calcium concentration are closely associated with numerous diseases, and consequently, calcium signaling is subject to stringent regulation [2].

Orai1 represents a vital calcium channel that mediates store-operated calcium entry (SOCE), the primary mechanism of calcium influx in non-excitable cells. SOCE is regulated by endoplasmic reticulum (ER) calcium concentration. The two main functional proteins involved in SOCE are Orai1 and STIM1. Orai1 is a four-transmembrane protein localized in the plasma membrane, composed of four transmembrane α-helices (TM1-4), three loop domains connecting the transmembrane regions, and N- and C-termini exposed to the cytoplasm [3], as illustrated in [Figure 1: see original paper]A. The crystal structure of Drosophila Orai protein has been resolved, revealing a hexameric assembly where six TM1 helices form the central ion channel pore [4]. STIM1 is a single-pass transmembrane protein localized in the ER, with two EF-hand domains in its N-terminus that sense calcium concentration changes [5]. Upon activation of G proteins

and tyrosine kinase receptors, phospholipase C (PLC) is activated, leading to hydrolysis of the specific phospholipid PIP2 in the plasma membrane to form IP3. IP3 rapidly binds to IP3 receptor channels on the ER, causing ER calcium release. This triggers STIM1 dimerization and migration to ER-plasma membrane contact sites, where the SOAR domain of STIM1 interacts with the C- and N-termini of Orai1, thereby activating the Orai1 calcium channel [6]. Orai1 activation participates in diverse cellular activities and has been implicated in multiple diseases, including severe combined immunodeficiency syndrome caused by the channel-inactivating mutation Orai1 R91W [7], heart failure and skeletal muscle weakness resulting from Orai1 deficiency [8], and various cancers such as breast, prostate, and colon cancers [9-12]. Therefore, elucidating the activation mechanism and function of Orai1 is crucial for developing therapeutic strategies for these diseases.

Liposomes are artificially prepared vesicles with a lipid bilayer structure and amphiphilic properties, widely applied in targeted drug delivery and as carriers for biomacromolecules [13]. Liposome reconstitution technology has been utilized to study membrane protein function. As a critically important class of proteins, membrane proteins directly participate in substance transport and signal transduction. Liposome reconstitution technology can effectively simulate the lipid environment of membrane proteins, eliminating interference from complex intracellular environments and enabling better functional studies [14-15]. Although the function of the calcium channel Orai1 has been extensively investigated, the influence of other intracellular molecules cannot be excluded. Purifying Orai1 protein and reconstituting it into liposomes to study its structure and function effectively eliminates the complex cellular environment, allowing for more direct investigation of its interacting proteins, channel activation mechanisms, and screening of Orai1-related drugs. Therefore, we employed liposome reconstitution technology to prepare Orai1 proteoliposome complexes, as schematically shown in [Figure 1: see original paper]B.

Materials and Methods

1.1 Materials

DNA polymerase, restriction endonucleases, and T4 DNA ligase were purchased from Thermo Fisher Scientific. Competent cells were from TransGen Biotech. Fura-2 was from Invitrogen. Ionomycin was from EMD Chemicals. GSH beads for protein purification were from GE Healthcare. Lipids POPC/POPS for liposome preparation were from Avanti. Bio-bead SM adsorbent beads were from Bio-Rad. 3C Protease and HA antibody were from Sigma-Aldrich.

1.2.1 Liposome Preparation

Three lipids dissolved in chloroform—phosphatidylcholine (POPC), phosphatidylserine (POPS), and rhodamine-labeled phosphatidylethanolamine (Rho-PE)—were mixed according to the proportions shown in , with a total

lipid concentration of 10 mmol/L. The mixture was carefully dried under nitrogen gas, then further dried under vacuum for 1 hour to remove residual chloroform. The dried lipids were resuspended in buffer (25 mmol/L Hepes, 100 mmol/L KCl) and subjected to 10 freeze-thaw cycles in liquid nitrogen. During hydration, multilamellar vesicles formed. These multilamellar vesicles were then extruded through a Mini-Extruder with polycarbonate membranes to form uniform large unilamellar vesicles (LUVs). Finally, the prepared liposomes were observed under a fluorescence microscope.

1.2.2 Molecular Cloning and Orai1 Protein Expression and Purification

The Orai1 gene sequence was amplified by PCR and subcloned into the pGEX-6P-1 vector via restriction enzyme digestion and ligation, with an HA tag sequence added at the C-terminus of Orai1. The GST-Orai1 fusion protein with HA tag was expressed in *E. coli* through in vitro induction. *E. coli* containing the Orai1 expression vector were cultured in LB medium at 37°C with shaking until OD_{600} reached 0.8, then induced with 0.5 mM IPTG at 24°C for 12 hours. Cells were harvested by centrifugation and lysed by sonication. Membrane fractions were obtained by ultracentrifugation at 32,000 rpm for 30 minutes. Membrane proteins were extracted with buffer containing 2% Triton X-100, and the supernatant containing GST-Orai1 was purified using Glutathione Sepharose beads. The protein was eluted with buffer containing 10 mM reduced glutathione and 0.1% Triton, further purified by size-exclusion chromatography, and concentrated to 1 mg/ml for subsequent experiments.

1.2.3 Reconstitution of Orai1 Protein into Liposomes

Purified GST-Orai1 fusion protein was mixed with liposomes at a molar ratio of 1:1000. Calcium-containing buffer was added to achieve a final Ca^{2+} concentration of 200 mol/L, followed by incubation at room temperature for 5 minutes. Bio-beads SM-2 were then added to the mixture five times at 1-hour intervals to adsorb Triton-X100, thereby reconstituting the membrane protein Orai1 into calcium-loaded liposomes. The reconstituted liposomes were collected by centrifugation at 14,000g for 10 minutes, and the supernatant was used for subsequent experiments.

1.2.4 Detection of Proteoliposome Reconstitution Efficiency

Thirty microliters of reconstituted Orai1 proteoliposomes or Orai1 protein alone (as control) were mixed with 100 L of 1.9 mol/L sucrose and placed at the bottom of a centrifuge tube. One hundred microliters of 1.25 mol/L sucrose and 20 L of buffer were sequentially layered on top to create a sucrose density gradient. Samples were centrifuged at 54,000 rpm for 1 hour using a Beckman TLS55 rotor, then divided into five 50 L fractions from top to bottom. Fifteen microliters from each fraction were analyzed by Western Blot.

1.2.5 Detection of Orai1 Protein Orientation in Liposomes

Twenty microliters of reconstituted Orai1 proteoliposomes were treated with 1 M 3C protease at 4°C overnight. After digestion, loading buffer was added and samples were analyzed by SDS-PAGE followed by Western Blot detection.

1.2.6 In Vitro Calcium Release Assay

Reconstituted liposomes were pelleted by ultracentrifugation at 54,000 rpm for 30 minutes. The supernatant was removed, and the pellet was resuspended in calcium-free buffer to remove residual calcium ions. Fura-2 was added to a final concentration of 50 mol/L, and 2 M calcium ionophore ionomycin was added to release Ca^{2+} from within the liposomes. Calcium concentration in the solution was measured using a microplate reader by detecting Fura-2 fluorescence emission at 510 nm with excitation at 340 nm/380 nm.

1.2.7 Structural Analysis of Orai1 Protein in Liposomes

Thirty microliters of reconstituted Orai1 proteoliposomes were incubated with 1% detergents SDS or Digitonin at 4°C for 1 hour, then gently layered onto a 5-25% (wt/vol) sucrose gradient. Samples were centrifuged at 174,000 g for 2 hours using a Beckman TLS55 rotor and divided into 14 fractions from top to bottom. Fifteen microliters from each fraction were analyzed by Western Blot.

Results

2.1 Liposome Preparation

Liposomes prepared by the freeze-thaw method contained the fluorescent dye rhodamine. Fluorescence microscopy observation confirmed successful liposome preparation, as shown in [Figure 2: see original paper].

2.2 Orai1 Expression Vector Construction and Protein Purification

The Orai1 gene fragment was amplified from human cDNA by PCR, as shown in [Figure 3: see original paper]A. The Orai1 gene was inserted into the prokaryotic expression vector pGEX-6P-1 via restriction enzyme digestion and ligation. Successful vector construction was confirmed by double enzyme digestion verification ([Figure 3: see original paper]B) and sequencing, followed by transformation into BL21 competent cells for protein expression.

The calcium channel Orai1 is a plasma membrane protein, making its purification more challenging than soluble cytoplasmic proteins. We constructed the prokaryotic expression vector pGEX-6p-1-Orai1 to express GST-Orai1 fusion protein in *E. coli*. The fusion protein was extracted from the cell membrane using the detergent Triton X-100 and purified by binding to glutathione agarose gel, followed by further purification using Superdex 200 size-exclusion

chromatography to obtain the target protein, as shown in [Figure 3: see original paper]C.

2.3 Orai1 Proteoliposome Reconstitution Efficiency

To directly study the function of the calcium channel Orai1 in vitro, we prepared liposomes to mimic cellular membranes and reconstituted GST-Orai1 protein into them. Reconstitution efficiency was assessed by a flotation assay, where reconstituted Orai1 proteoliposomes were placed at the bottom of a sucrose density gradient and subjected to ultracentrifugation to determine whether Orai1 protein could float to the top of the gradient along with the liposomes. Purified GST-Orai1 fusion protein served as a control, as shown in [Figure 4: see original paper]A. The results demonstrated that GST-Orai1 protein, after reconstitution with liposomes, efficiently floated to the top of the sucrose gradient, whereas GST-Orai1 protein alone could not float to the top and instead sedimented to the lower portion of the gradient. These findings indicate successful reconstitution of the Orai1 fusion protein into liposomes, as shown in [Figure 4: see original paper]B.

2.4 Detection of Orai1 Protein Orientation in Liposomes

In the cell membrane, the N- and C-termini of Orai1 protein are located intracellularly to participate in protein interactions and activation, with calcium ions flowing from extracellular to intracellular space. Therefore, we needed to determine the orientation of Orai1 in liposomes. Using the GST and HA tags at the N- and C-termini of the Orai1 fusion protein, we performed a protease cleavage assay. If Orai1 were oriented as shown in [Figure 5: see original paper]A, with N- and C-termini facing outward from the liposome interior, 3C protease would cleave the GST tag. Experimental results showed that GST-Orai1 protein in proteoliposomes could be cleaved by 3C protease, resulting in a smaller molecular weight band ([Figure 5: see original paper]B), indicating that the N- and C-termini of the Orai1 calcium channel were exposed on the outer surface of the liposomes. Consequently, calcium ion flow should occur from inside to outside the liposomes, requiring calcium ions to be encapsulated within the liposomes during reconstitution.

2.5 Orai1 Proteoliposome Calcium Ion Release Assay

We assessed calcium ion encapsulation within liposomes by adding ionomycin, which induces calcium release from liposomes. The calcium-sensitive dye Fura-2 was used to detect changes in fluorescence intensity ratio at 340 nm/380 nm excitation, as shown in [Figure 6: see original paper]. The results demonstrated that ionomycin effectively released calcium ions from within the liposomes, confirming intact calcium packaging. This validates the system for subsequent functional studies.

2.6 Orai1 Structure and Function Analysis

Following confirmation of reconstitution efficiency and orientation, we investigated whether Orai1 could form oligomers to function as a calcium channel. We solubilized Orai1 proteoliposomes using two detergents: SDS and Digitonin. SDS is a strong ionic detergent that dissociates protein oligomers, whereas Digitonin is a mild detergent that extracts proteins from membranes while preserving their oligomeric structure. After solubilization, proteoliposomes were subjected to sucrose density gradient centrifugation and separated into 14 fractions. Protein standards were used to determine molecular weights at different gradient positions, as shown in [Figure 7: see original paper]. Western blot analysis revealed that SDS-treated Orai1 protein primarily localized to the upper fractions, while Digitonin-treated Orai1 protein was concentrated in the lower fractions. These results demonstrate that Orai1 protein can form oligomeric structures in liposomes capable of functioning as a calcium channel.

Discussion

Membrane proteins play essential roles in various cellular activities, including substance transport, signal transduction, catalytic reactions, and membrane shaping. Membrane proteins constitute approximately 20-30% of the entire proteome, with 60% serving as drug targets, necessitating deeper investigation of their physiological functions. However, because membrane proteins are embedded in lipid bilayers and influenced by the complex intracellular environment—including intricate metabolic pathways and protein-protein interactions—direct functional studies are challenging [16]. Although biophysical and biochemical methods can isolate membrane proteins from membranes, they often fail to maintain normal structure and function when removed from their native lipid environment. Liposome reconstitution technology represents an innovative approach for investigating membrane protein characteristics and functions, offering the advantage of studying membrane proteins in a native-like lipid environment while enabling encapsulation of small molecules to investigate transmembrane transport functions.

Store-operated calcium channel Orai1 is localized in the plasma membrane and represents the primary calcium influx pathway in non-excitable cells, making it directly relevant to numerous physiological functions. Elucidating its activation mechanism is therefore critically important. We sought to establish an in vitro research system for Orai1 using liposome reconstitution technology. We first prepared liposomes in vitro ([Figure 2: see original paper]) and expressed and purified the Orai1 fusion protein GST-Orai1-HA ([Figure 3: see original paper]C). Membrane protein purification is more difficult than soluble protein purification, requiring mild detergents for extraction. We tested multiple detergents including Triton X-100, DM, DDM, OG, and LDAO, ultimately selecting Triton X-100 for membrane protein extraction. GST affinity chromatography and size-exclusion chromatography were used to purify Orai1 protein. Once removed from the stabilizing lipid environment, membrane proteins are prone

to denaturation and precipitation. By mixing Orai1 protein with liposomes at an appropriate ratio and gradually removing detergent, we achieved membrane protein reconstitution. Sucrose density gradient centrifugation confirmed successful reconstitution, as proteoliposomes have lower density and float to the top of the gradient during centrifugation ([Figure 4: see original paper]B).

Membrane protein orientation in liposomes is another critical parameter requiring detection and control. Fluorescence quenching assays can determine orientation by coupling fluorophores to membrane proteins; if fluorophores are inside liposomes, they are protected from quenchers, thereby revealing protein orientation, which is crucial for understanding transport direction, ion channel polarity, or reaction orientation [17]. Our purified fusion protein GST-Orai1 contains a 3C protease cleavage site between the GST tag and Orai1 protein, enabling orientation determination through proteolysis. The cleavage of the GST tag ([Figure 5: see original paper]B) demonstrated that Orai1 N- and C-termini were exposed on the liposome exterior, indicating calcium ions should flow from inside to outside the liposomes. Therefore, we included calcium ions in the reconstitution system, which became encapsulated within liposomes upon completion. Ionomycin-mediated calcium release and detection with Fura-2 ([Figure 6: see original paper]) confirmed intact calcium packaging.

The functional competence of in vitro expressed and purified Orai1 depends on its ability to form oligomeric calcium channels. While Drosophila Orai forms hexameric channels, the human Orai1 structure remains unresolved. Some biochemical studies suggest that tetrameric Orai1 exhibits higher calcium selectivity, consistent with endogenous store-operated calcium channels [18], though the precise oligomeric state awaits structural determination. Nevertheless, Orai1 must form oligomers with its first transmembrane domain constituting the calcium channel pore. We exploited the different properties of SDS and Digitonin detergents to solubilize proteoliposomes: SDS denatures and dissociates proteins, whereas Digitonin is a mild detergent that preserves oligomeric structures. Sucrose density gradient centrifugation can distinguish Orai1 proteins in different oligomeric states ([Figure 7: see original paper]), confirming that Orai1 forms oligomers in liposomes capable of functioning as a calcium channel.

In summary, our method successfully transferred the calcium channel Orai1 from the cell membrane to a similar lipid environment in liposomes while eliminating complex intracellular factors. This system will facilitate direct investigation of interacting proteins such as STIM1 and calmodulin, including their interaction mechanisms, domains, and sites. It will also enable elucidation of activation mechanisms, such as whether Orai1 can be directly activated by STIM1. Additionally, it allows study of important Orai1 residues, such as reconstituting the mutant Orai1 R91W protein to investigate its activation mechanism, and supports research and development of Orai1 channel inhibitors and activators, including mechanism studies of 2-APB, SKF-96365, and other small molecules. Overall, this study establishes a novel method for investigating store-operated calcium channel Orai1 and provides new insights for studying other membrane

proteins.

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Note: Figure translations are in progress. See original paper for figures.