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Expression, purification and structural characterization of the type 1-specific ATP binding site of IP_3 receptor (IP_3R1-ATPA)

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ABSTRACT

The inositol 1,4,5-triphosphate receptor (IP_3R), an IP_3-gated Ca^{2+} release channel on the endoplasmic reticulum (ER) membrane, plays a critical role in maintaining cytosolic Ca^{2+} homeostasis in cells. Particularly, ATP increases IP_3R activity by binding to ATPA, a putative glycine-rich Walker A-type motif (GXGXXG) specific to type 1 IP_3R (IP_3R1). Here, we established an efficient process to produce the ATPA containing domain of IP_3R1 (IP_3R1-ATPA) using a chaperone co-expression system in *Escherichia coli*. The recombinant protein was well expressed as a soluble form and showed a high thermostability. Circular dichroism results indicated a mainly α-helical conformation of the purified protein. Additionally, model structures of IP_3R1-ATPA were calculated and validated using different modeling algorithms. The structural models of IP_3R1-ATPA not only supported the observed high thermostability, but also suggested a potential ATP binding site.

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1. Introduction

Inositol-1,4,5-triphosphate (IP_3) receptor (IP_3R) is an intracellular Ca^{2+} release channel that is induced by the second messenger IP_3 [1]. The IP_3R consists of three regions (N-terminal region, regulatory region, and C-terminal region) (Fig. 1A). The N-terminal region encompasses suppressor domain (SD) and IP_3-binding core domain (IBC), which are responsible for IP_3 binding and also involved in channel gating. The regulatory domain interacts with various proteins and modulators, and the C-terminal region contains transmembrane domain (TMD).

The IP_3Rs are encoded by three different genes, giving rise to IP_3R1, IP_3R2, and IP_3R3, which have 60–80% homology at the amino acid level [2]. Functional differences between the three isoforms in the regulation of IP_3-induced Ca^{2+} release (IICR) by IP_3 and several modulators, such as adenosine triphosphate (ATP), have been reported [3–6]. Although ATP is not essential for channel gating, it affects the activity of IP_3R in an allosteric fashion. ATP influences the channel open probability and modulates IICR in the presence of activating concentrations of IP_3 and Ca^{2+} [5,6]. ATP binds to specific ATP-binding regions, including glycine-rich motifs (consensus sequence GXGXXG), and the IP_3R sequence contains several such motifs [7]. One site, called ATPA (residues 1772–1777 in rat IP_3R1), is unique to IP_3R1, while a second site, ATPB (residues 2015–2020 in rat IP_3R1), is common to all three isoforms [8] (Fig. 1A). Analogs of ATP, such as caffeine, adenophostin A, and cyclic ADP-ribose, also interact with IP_3R. Although adenophostin A and cyclic ADP-ribose do not interact with the ATP-binding regions, caffeine binds these regions directly [7,9].

To investigate the ATP binding mechanisms of IP_3R at the molecular level, large-scale preparation and purification of a protein containing the ATP binding sites is essential. *Escherichia coli* is a preferred host for protein expression because of several benefits, including easy transformation, rapid growth, inexpensive equipment for growth and storage, and well-characterized biology and genetics. However, high expression of recombinant proteins

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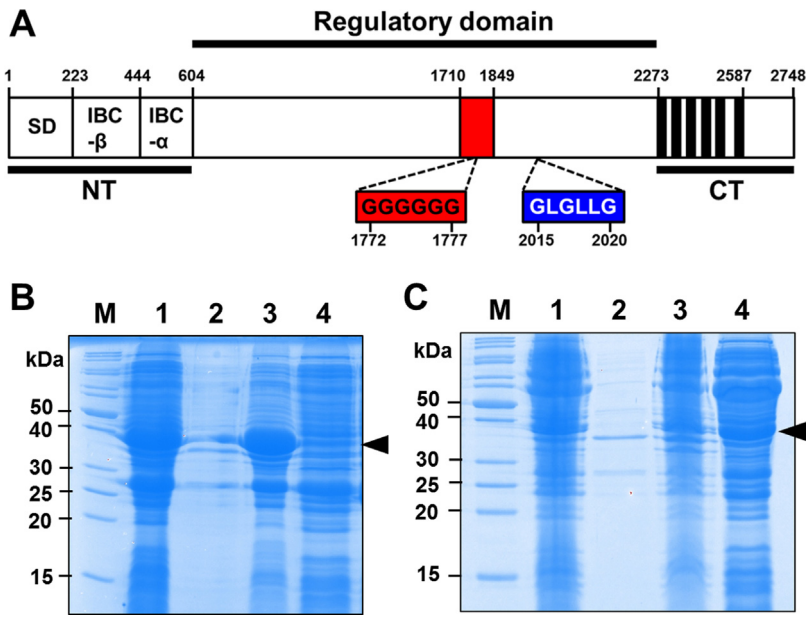

Fig. 1. Schematic representation of IP_3R1 and the expression and purification of IP_3R1-ATPA. (A) Schematic diagram of the functional domains in the primary sequence of IP_3R1. Amino acid sequences of the first ATP binding site (GGGGGG) and the second ATP binding site (GLGLLG) are represented in red and blue boxes, respectively. The construct of IP_3R1-ATPA used in this study (residues 1772–1777 in rat IP_3R1) are represented as red square. Transmembrane domain (TMD) is depicted as black bars. SD, suppressor domain; IBC, IP_3-binding core domain; NT, N-terminal region; CT, C-terminal region. (B, C) SDS-PAGE analysis of the expression and solubility profiles of IP_3R1-ATPA alone at 15 °C (B) and IP_3R1-ATPA co-expressed with chaperone plasmid pG-KJE8 (C). Lane M, protein molecular weight marker; lanes 1 and 2, pellet and supernatant of lysate before sonication; lanes 3 and 4, pellet and supernatant of lysate after sonication. The estimated molecular weight of GST-fused IP_3R1-ATPA was approximately 40 kDa and the target bands were represented by black arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

often results in inaccurate folding, aggregation, and inclusion body formation. Many attempts have been made to overcome these problems, and one of the most extensively used methods is co-expression of molecular chaperones. Co-expression with molecular chaperones in *E. coli* can facilitate the refolding of misfolded and aggregated proteins, thereby increasing the solubility and decreasing the aggregation of recombinant proteins [10,11].

Here, we prepared a recombinant protein comprising residues 1710–1849 of rat IP_3R1 (IP_3R1-ATPA), which includes the first ATP binding site, and co-expressed the protein in glutathione S-transferase (GST)-fused form with molecular chaperones in *E. coli*. Solubilized and purified proteins were used to characterize the structural properties of IP_3R1-ATPA.

2. Materials and methods

2.1. Preparation of the IP3R1-ATPA construct

The gene coding region containing the first ATP binding site of IP_3R1 from *Rattus norvegicus* (residues 1710–1849, hereafter referred to as IP_3R1-ATPA) was amplified by polymerase chain reaction (PCR) using the full-length IP_3R1 gene as a template. The PCR products were digested with BamHI and XhoI restrictions enzymes and inserted into the pGEX-4T-3 vector (GE Healthcare). The ligation mixture was transformed into *E. coli* DH5α cells, and the construct was verified by DNA sequencing using a high-throughput DNA analyzer (Bioneer Inc.). The sequence identity and similarity between rat IP_3R1-ATPA and human IP_3R1-ATPA are 94.9% and 97.9%, respectively.

2.2. Co-expression of IP3R1-ATPA with molecular chaperones

E. coli BL21-CodonPlus (DE3) cells transformed with the constructed plasmid were grown at 37 °C in sterilized Luria Broth (LB) medium containing 100 μg/ml ampicillin. In order to compare the yields of IP_3R1-ATPA in different media, the cells were also grown in sterilized SOB (Super Optimal Broth) and TB (Terrific Broth). IP_3R1-ATPA was expressed in GST-fused form at 37 °C for 4 h or at 15 °C for 16 h. In both cases, the final concentration of isopropyl-β-D-1-thiogalactopyranoside (IPTG) was 0.5 mM.

To co-express IP_3R1-ATPA and chaperones, cells were transformed in two steps: transformation of *E. coli* BL21 with a chaperone plasmid followed by transformation with the constructed plasmid. Five commercially available plasmids, pGro7, pKJE7, pTf16, pG-Tf2, and pG-KJE8, encoding molecular chaperones were used (Supplementary Table 1). The transformant was inoculated into LB medium containing 20 μg/ml chloramphenicol and 50 μg/ml ampicillin for plasmid selection, and 1.5 mg/ml L-arabinose and/or 10 ng/ml tetracycline were added to induce chaperone expression at 37 °C with shaking. IPTG was added to a final concentration of 0.5 mM at an OD_{600} of approximately 0.6. Cells were grown for an additional 16 h at 15 °C and were harvested by centrifugation at 7077 × *g* for 20 min.

2.3. Purification of IP_3R1-ATPA

The cell pellet from the *E. coli* cell culture after co-expression of chaperones was resuspended in lysis buffer (20 mM Tris–HCl, pH 8.4, 10% glycerol, 0.2% NP-40, 500 mM NaCl, 10 mM 2-mercaptoethanol, and 0.2 mM TCEP). Cells were lysed on ice with sonication (duration time: 1 min, duty time: 40% with 2-min cooling) using a Branson Sonifier cell disruptor (Hielscher). After centrifugation, the supernatant from the cell lysates was loaded onto a GSTrap™ FF (GE Healthcare) column. The fusion protein was eluted with elution buffer (20 mM Tris–HCl, pH 8.4, 5% glycerol, 300 mM NaCl, 2 mM DTT, 10 mM glutathione). The GST tag was cleaved from IP_3R1-ATPA by the addition of thrombin, and the solution was dialyzed against binding buffer at 4 °C for 16 h. The digested sample was applied to a GSTrap™ FF column to remove the uncleaved fusion protein, the separated GST tag fragment, and

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