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Expression and purification of a difficult sarcomeric protein: Telethonin

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ABSTRACT

Telethonin anchors the N-terminal region of titin in the Z-disk of the sarcomere by binding to two immunoglobulin-like (Ig) domains (Z1 and Z2) of titin (Z1Z2). Thereby telethonin plays an important role in myofibril assembly and in muscle development and functional regulation. The expression and purification of recombinant telethonin is very challenging. In previous studies, recombinant telethonin expressed from *E. coli* was refolded in the presence of Z1Z2. Here, we report various strategies to establish a reliable and efficient protocol for the preparation of telethonin and titin Z1Z2 protein. First, a co-expression strategy was designed to obtain soluble Z1Z2/telethonin complexes. The concentration of antibiotics and the type of expression vector were found to be important for achieving high yields of purified complex. Second, the five cysteine residues of telethonin were mutated to serine to avoid severe problems with cysteine oxidation. Third, a short version of telethonin (telethonin$_{1\text{-}90}$) was designed to avoid the proteolytic degradation observed for longer constructs of the protein. The short telethonin formed a highly stable complex with Z1Z2 with no degradation being observed for 30 days at 4 °C. Fourth, an improved refolding protocol was developed to achieve high yields of Z1Z2/telethonin complex. Finally, based on the crystal structure in which Z1Z2 and telethonin$_{1\text{-}90}$ assemble into a 2:1 complex, a single chain fusion protein was designed, comprising two Z1Z2 modules that are connected by flexible linkers N- and C-terminally of the telethonin$_{1\text{-}90}$. Expression of this fusion protein, named Z1Z, affords high yields of soluble expressed and purified protein.

1. Introduction

The muscle Z-disk defines the border between adjacent sarcomere units in the myocyte cytoskeleton. In the Z-disk at least three sarcomere filament systems are anchored and aligned, comprising titin, actin and nebulin [1]. The giant muscle protein titin is the largest protein known with a molecular weight of up to 4 MDa [2,3], spanning one half of the sarcomere in striated and cardiac muscle [4]. In the Z-disk, two titin molecules are assembled into a complex involving interactions of the two N-terminal immunoglobulin-like (Ig) domains Z1 and Z2 (Z1Z2). The small 167-residue protein telethonin was found to mediate this assembly [5]. Telethonin is one of the most abundant transcripts in skeletal muscle [6,7]. In the Z-disk, many proteins have been found to interact strongly with telethonin, such as the muscle LIM protein (MLP), the potassium channel regulation protein Mink, the FATZ protein and the muscle growth factor myostatin [4]. Mutations in the telethonin genes are associated with limb-girdle muscular dystrophy type 2G (LGMD 2G), as well as hypertrophic and dilated cardiomyopathy [8]. These findings demonstrate that telethonin plays an important role in myofibril assembly and in muscle development and functional regulation [9].

The Z1Z2/telethonin complex has been suggested to be critical for the Z-disk structure and for anchoring titin in the Z-disk [10]. Biochemical studies showed that the Z1Z2/telethonin complex is required for sarcomere integrity and muscle growth [11]. Biochemical data and the crystal structure of the Z1Z2/telethonin

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complex reported by Zou et al. [5] revealed that the titin Z1Z2 protein is assembled into an antiparallel (2:1) sandwich complex with telethonin. The structural integrity of the Z-disk depends upon the tight network of hydrogen bonds between Z1Z2 and telethonin. These bonds enable the complex to resist extremely high mechanical loads. Molecular dynamics simulations and single-molecule force spectroscopy experiments demonstrated that the Z1Z2/telethonin complex resists large mechanical force (about 700 pN) by β strand crosslinking, in contrast to the relatively low mechanical force (about 168 pN) that Z1Z2 can resist without telethonin. The interaction between Z1Z2 and telethonin represents the strongest protein-protein interaction observed so far [12,13]. In this respect, the complex is perfect for firmly anchoring the giant muscle protein titin in the Z-disk [4,14].

Previous studies indicated that telethonin may exist in distinct conformations, depending on whether or not it is bound to Z1Z2 and further indicated that the isolated telethonin is not stable [4]. These results are consistent with the observation that purified telethonin tends to aggregate in solution, and that telethonin is usually expressed into inclusion bodies [9]. It is thus believed that Z1Z2 plays a role in stabilizing the fold of telethonin [4]. The Z1Z2/telethonin complex cannot be formed by simply mixing the two components. Z1Z2 preferentially adopts semi-extended conformations in solution, with close-hinge arrangements [15], while in the complex with telethonin Z1Z2 adopts a fully extended conformation [9]. This conformation probably results from an induced fit of Z1Z2 upon binding to its partner. The predicted secondary structure of free telethonin is completely different than the secondary structure observed in the complex. This indicates that complex formation involves a mutually induced conformational change [4,15]. The refolding protocol provided an effective method to quickly obtain large amounts of the protein previously used for the structural biology studies [4,15]. The improved refolding protocol is described here in detail. The yeast hybrid carried out by Gautel et al. was the first to prove that Z1Z2 and telethonin could bind to each other [16]. Since then, the binding experiment had not been reproduced until the co-expression experiment was carried out in our lab, but these data have not been published until now. In this paper several new protocols are established to achieve reliable and stable complex sample for different research purposes.

Therapeutic peptides and proteins have attracted considerable interest as they exhibit high binding affinity, high specificity, high solubility and low toxicity. The short half-life of many peptides and proteins, typically from a few minutes to a few hours resulting from the enzymatic degradation and renal clearance, hampers the application of these peptides and proteins in clinical treatment [17]. In order to prolong the half-life of these peptides and proteins, various strategies have been proposed [18]. Genetically encoded fusion of a peptide or protein to a larger protein to increase the size is also widely used to prolong the retention time in circulation. Different fusion tags have been proposed, such as proteins with naturally long half-lives. These proteins include human serum albumin or the Fc domain of IgG, or non-natural polypeptide stretches, such as XTEN [19], PAS [20], and HAP [21]. The molecular weight of the Z1Z2/telethonin complex is about 50 kDa and thus beyond the threshold of the renal filtration (in the range of 40–50 kDa) [22]. The protein complex is highly stable in plasma. Therefore, the Z1Z2/telethonin complex can be used as a scaffold to increase the stability of therapeutic peptides and proteins by genetic fusion. We established single-chain construct comprising two Z1Z2 regions flanking an N-terminal fragment of telethonin which could be highly efficiently expressed as a stable fusion protein, termed ZTZ. The ZTZ protein could be beneficial for further research on therapeutic applications.

2. Materials and methods

2.1. Strains and plasmids

All plasmids were constructed in *E. coli* strain DH5α. The sequences of all cloned PCR products and mutants were confirmed by sequencing (Invitrogen, Beijing, China). *E. coli* BL21(DE3) was used as the expression host. The expression plasmids pET-3d, pET-6d, pET-24d and pACYC-9d were obtained from Novagen (Madison, WI). The pACYCM-9d was modified from pACYC-9d by removing the His-tag. Primers were ordered from Sangon Biotechnology (Shanghai, China). Isopropylthio-β-D-galactoside (IPTG) was purchased from Sigma-Aldrich (Shanghai, China).

A construct comprising the two N terminal Ig domains of titin (residues 1–196) Z1Z2 was cloned into pET-3d and pET-6d vectors. In pET-3d, Z1Z2 is fused to the His_6-tag directly. In pET-6d, a TEV protease cleavage site exists between the His_6-tag and the Z1Z2 gene. Different regions of telethonin (residues 1–167 and residues 1–90) and cysteine mutants were cloned into pET-24d, pACYC-9d and pACYCM-9d.

2.2. Site-directed mutagenesis

The QuickChange™ site-directed mutagenesis protocol (Stratagene, La Jolla, CA) was used to introduce point mutations into $telethonin_{1\text{-}167}$ (C38S, C57S, C127S) using mutagenic oligonucleotides from 30 to 35 bases in length (Sangon Biotechnology, Shanghai, China). C8 and C15, which are close to the N-terminus of telethonin, were mutated into serines by PCR. A 49-mer primer with two point mutations and an N-terminal cloning site (NcoI) (5′-ATCCATGGCTACCTCAGAGCTGAGCAGCAGCGAGGTGTCGGAGGA-GAAC-3′) and the C-terminal cloning primer (5′-AAAGGTACCT-TAGCCTCTCTGTGCTTCCTGG-3′) with a *Kpn*I restriction site were designed for the point mutation of C8 and C15. The PCR fragment was cut by the restriction enzymes *Nco*I and *Kpn*I and cloned into the expression vectors pACYC-9d. The telethonin with no cysteine was referred to as $telethonin_{1\text{-}167M}$, which was different from the $telethonin_{1\text{-}167}$ with 5 cysteines. The N-terminal truncation of $telethonin_{1\text{-}90}$ without cysteines was produced by PCR using the primer-F (CGCCATGGCTACCTCAGAGCTG) containing a *Nco*I restriction site and primer-R (TCGGATCCGGTACCTTACGGCA) containing a *Bam*HI restriction site. The PCR fragment was cut by the restriction enzymes *Nco*I and *Bam*HI, and cloned into the expression vectors pACYC-9d and pET-24d.

2.3. Expression and purification

The plasmids containing the genes encoding the Z1Z2 and $telethonin_{1\text{-}167}$ with a N-terminal His_6-tag were transformed into *E. coli* BL21 (DE3). A single colony was picked randomly and cultured in LB medium with 50 μg/ml kanamycin or 100 μg/ml ampicillin overnight at 37 °C. For the co-expression, different pairs of the plasmids were co-transformed into *E. coli* BL21(DE3) and were grown in LB medium with 100 μg/ml ampicillin and 50 μg/ml kanamycin. Overnight cultures were grown in LB medium at 37 °C, diluted 50-fold, and grown until the optical density of 0.4–0.6 at 600 nm was reached. Then IPTG was added with a final concentration 1 mM and the bacteria were cultured for an additional 6 h at 30 °C. The cells were harvested by centrifugation at 5000 rpm for 20 min at 4 °C, resuspended in lysis buffer (25 mM Tris/HCl, 300 mM NaCl, pH 8.0, supplemented with 1 mg/ml lysozyme and 0.01 mg/ml DNase I), and lysed by pulsed sonication (5 min, 40% power, large probe, Fisher Scientific model 550) followed by centrifugation at 15000 rpm for 1 h.