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Research paper

Complete primary structure of the I-band region of connectin at which mechanical property is modulated in zebrafish heart and skeletal muscle

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ABSTRACT

Connectin, also called titin, is the largest protein with a critical function as a molecular spring during contraction and relaxation of striated muscle; its mutation leads to severe myopathy and cardiomyopathy. To uncover the cause of this pathogenesis, zebrafish have recently been used as disease models because they are easier to genetically modify than mice. Although the gene structures and putative primary structures of zebrafish connectin have been determined, the actual primary structures of zebrafish connectin in heart and skeletal muscles remain unclear because of its large size and the PCR amplification-associated difficulties. In this research, using RT-PCR amplification from zebrafish heart and skeletal muscles, we determined the complete primary structures of zebrafish connectin in the I-band region at which mechanical property is modulated by alternative splicing. Our results showed that the domain structures of zebrafish connectins were largely similar to those of human connectins; however, the splicing pathways in the middle-Ig segment and the PEVK segment were highly diverse in every isoform. We also found that a set of 10 Ig domains in the middle-Ig segment of zebrafish connectin had been triplicated in human connectin. Because these triplicate regions are expressed in human leg and diaphragm, our findings may provide insight into the establishment of walking with limbs and lung respiration during tetrapod evolution.

1. Introduction

Connectin, also called titin, is the largest protein that connects the *Z*-line to the M-line of the sarcomere in heart and skeletal muscle and maintains the sarcomere during muscle contraction and relaxation (Maruyama et al., 1976; Maruyama, 1976; Wang et al., 1979). In particular, the A-band region of connectin binds to thick filaments, and its I-band region functions as a molecular spring that keeps thick filaments at the center of the sarcomere during muscle relaxation. Mutation of connectin leads to several heart diseases and muscular disorders, such as dilated cardiomyopathy, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy in heart and hereditary myopathy with early respiratory failure, tibial muscular dystrophy, limb girdle muscular dystrophy, and centronuclear myopathy in skeletal muscles (Linke and Hamdani, 2014).

The domain structure of human connectin is mainly divided into the *Z*-line region, the I-band region, and the A-band region (Labeit and Kolmerer, 1995). The N-terminus *Z*-line region includes two Ig domains and some Z-repeats, which bind to t-cap/telethonin and α-actinin, respectively, followed by seven Ig domains.

The I-band region is divided into a proximal-Ig segment, Novex I and II, an N2B segment, a middle-Ig segment, an N2A segment, a PEVK segment, and a distal-Ig segment. Of these segments, the proximal-Ig segment, comprising 15 Ig domains, and the distal-Ig segment, comprising 23 Ig domains, are constitutively expressed. Conversely, the following are alternatively spliced: Novex I and II (comprising an Ig domain and a unique sequence), the N2B segment (comprising 3 Ig domains and a unique sequence), the middle-Ig segment (comprising 53 Ig domains), the N2A segment (comprising 4 Ig domains and a unique sequence), and the PEVK segment (comprising a unique sequence in which >70% of the amino acids are proline, glutamate, valine, and lysine). As for the mechanical modulation of connectin, it has been recognized that the PEVK and N2B segments play important roles as molecular springs (Watanabe et al., 2002).

The A-band region is separated into the A-I junction segment, the D-zone, the C-zone, the P-zone, and the M-line segment. Among these segments, the A-I junction segment includes 2 Ig and 11 FN3 domains, the D-zone consists of 6 copies of 7-domain repeats (Ig-Fn-Fn-Ig-Fn-Fn-Fn),

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; Ig domain, immunoglobulin domain; FN3 domain, fibronectin type 3 domain; SH3 domain, Src homology 3 domain.

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the C-zone consists of 11 copies of 11-domain repeats (Ig-Fn-Fn-Ig-Fn-Fn-Fn-Ig-Fn-Fn-Fn), the P-zone includes 4 Ig and 3 Fn3 domains and a kinase domain, and the C-terminus M-line segment includes 10 Ig domains and unique sequences. The A-band region is highly conserved among chordates (Ohtsuka et al., 2011; Hanashima et al., 2012) and binds to the thick filaments.

The pathogenesis of myopathy and cardiomyopathy caused by mutations of human connectin has been investigated with animal models. Because of the phylogenetic relatedness, genetically engineered mice, in which each segment of connectin was partially deleted, have been used for the analysis of myopathies (Gotthardt et al., 2003 (M-line); Radke et al., 2007 (N2B); Granzier et al., 2009 (PEVK); Granzier et al., 2014 (A-I), Buck et al., 2014 (Ig3–9)). Despite the usefulness of mouse models, the considerable cost of investigating multiple candidate sites, which is attributed to the huge size of the connectin molecule, hampered progress in this research area. Recently, zebrafish have also been used as animal models as an alternative to mice for investigating connectin-related myopathy and cardiomyopathy (Xu et al., 2002, Seeley et al., 2007, Steffen et al., 2007, Myhre et al., 2014, Zou et al., 2015). Besides its economic benefit, the superiority of zebrafish models, such as their developmental speed and prolificacy, can accelerate research on connectin-related diseases. Therefore, it is important to reveal the complete primary structure of the I-band region, at which the mechanical property of the connectin molecule is modulated, in zebrafish.

While humans and mice have one connectin/titin gene, zebrafish have two, named TTNa and TTNb. The gene structures of zebrafish TTNa and TTNb are similar to that of human connectin in the Z-line, A-band, and M-line regions, but differ somewhat in the I-band region (Seeley et al., 2007). The primary structure of zebrafish connectin has been partially determined by RT-PCR (Seeley et al., 2007), but the complete primary structure and splicing isoforms remain unclear. In this study, we determined the complete primary structure of the I-band region of connectin, at which the mechanical property is modulated, by alternative splicing in zebrafish heart and skeletal muscle, and performed comparisons to human connectin. Moreover, we found that three sets of 10 Ig domains in the middle-Ig segment of human connectin (Witt et al., 1998) are derived from a set of 10 Ig domains in zebrafish connectin. The longest middle-Ig segment, which has all 53 Ig domains and includes three sets of 10 Ig domains, is expressed in soleus and diaphragm (Labeit and Kolmerer, 1995), which may provide insight into the evolution of limbs for walking and lung respiration.

2. Materials and methods

2.1. RT-PCR

Total RNAs were isolated from heart and skeletal muscle (back muscle) of young adult zebrafish using TRIzol reagent (Thermo Fisher

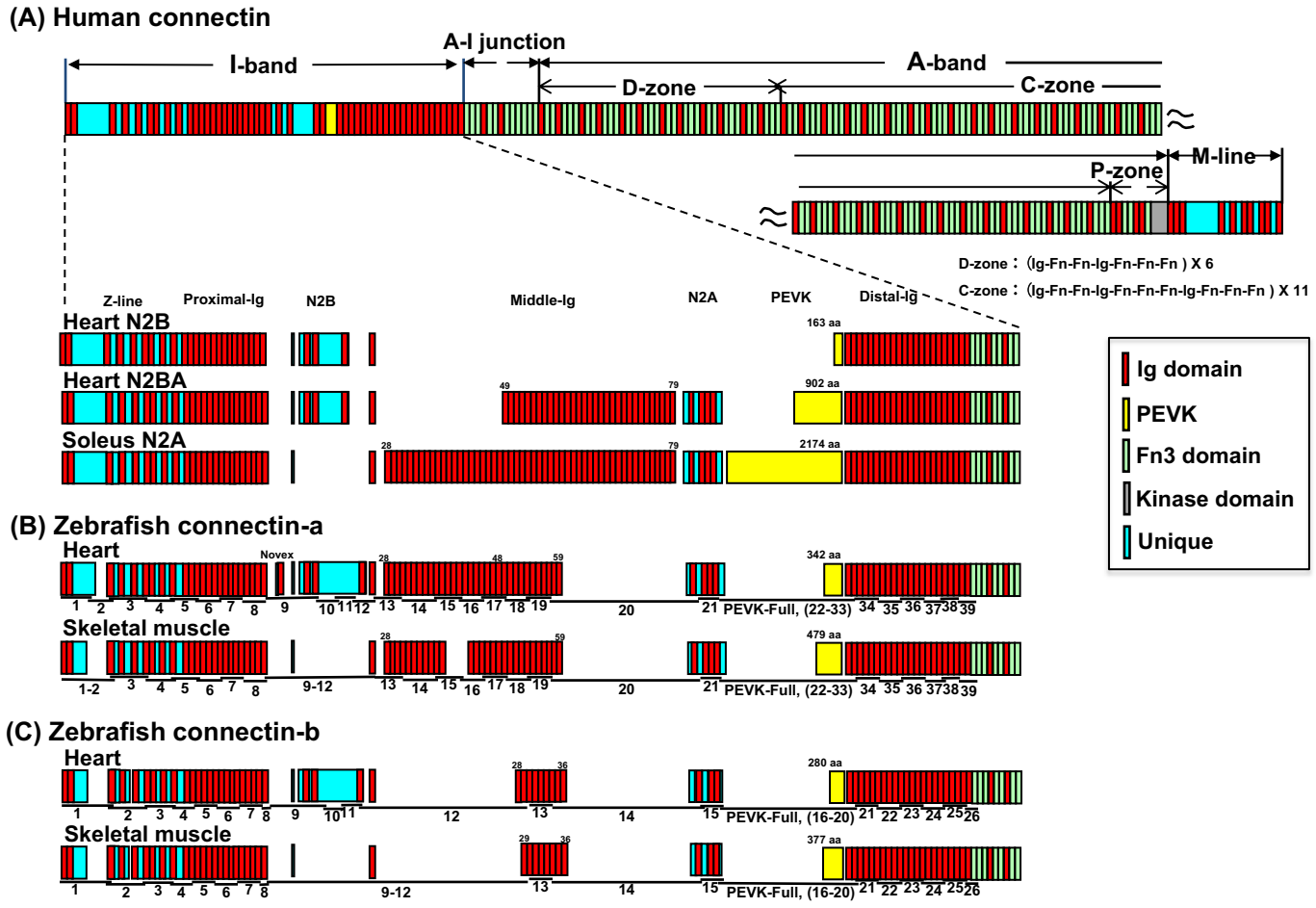

Fig. 1. Domain structures of zebrafish connectin in the I-band region of heart and skeletal muscle. (A) Human connectin. Upper: Full domain structure of human heart N2B isoform. Lower: Representative domain structures of the I-band region in heart N2B, heart N2BA, and skeletal muscle N2A isoforms of human connectin. (B) Zebrafish connectin-a. Representative isoforms in heart (DDBJ/EMBL/GenBank LC168151) and skeletal muscle (LC168153) are displayed. (C) Zebrafish connectin-b. Representative isoforms in heart (LC168152) and skeletal muscle (LC168154) are displayed. Numbers in (B) and (C) indicate the PCR fragments amplified in this research (see Supplementary material). Red, Ig domain. Yellow, PEVK segment. Green, FN3 domain. Gray, Kinase domain, Blue, Unique sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)