Abnormal junctional membrane structures in cardiac myocytes expressing ectopic junctophilin type 1

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Abstract **Recent studies indicate that junctophilin (JP) subtypes contribute to the formation of the junctional membrane complexes between the plasma membrane and the endoplasmic/sarcoplasmic reticulum (ER/SR) in excitable cells. Cardiac muscle contains the diad, in which the transverse (T) tubule of the invaginated cell membrane is closely associated with the SR membrane, and skeletal muscle bears the triad, in which the T-tubule is associated with two SR membranes on the both sides. Among defined JP subtypes, JP-2 is specifically expressed in cardiac muscle, while skeletal muscle cells contain both JP-1 and JP-2. These observations, together with other findings, suggest that the triad might be constructed in a JP-1-dependent manner after the achievement of JP-2-mediated diad formation during skeletal muscle maturation. In this study using transgenic mice, we examined whether the triad can be formed when JP-1 is additionally expressed in cardiac muscle. Immunochemical analysis demonstrated co-expression of JP-1 and JP-2 in cardiac myocytes from the transgenic mice. In cardiac muscle expressing JP-1, abnormal junctional membranes were frequently observed under the electron microscope, in which the T-tubules were rolled up with the SR membranes at several turns, but authentic triad formation could not be detected. Therefore, ectopic JP-1 expression cannot convert the diad to the triad in cardiac myocytes. The present results suggest that triad formation requires an as yet unknown skeletal muscle-specific mechanism, in addition to the JP subtypes.**

Key words: Diad; Junctophilin; Sarcoplasmic reticulum; Striated muscle; Transverse tubule; Triad

1. Introduction

During excitation–contraction (E-C) coupling of striated muscles, the signal conversion of depolarization into an increase in cytoplasmic Ca^{2+} concentration occurs at the junctional membrane complex between the transverse (T) tubule of invaginated plasma membrane and the sarcoplasmic/endoplasmic reticulum (SR/ER) functioning as the intracellular Ca^{2+} store. In Ca^{2+}-induced Ca^{2+} release in cardiac muscle, Ca^{2+} flows through the voltage-gated L-type Ca^{2+} channel, binds to open the ryanodine-sensitive Ca^{2+} release channel (ryanodine receptor) and triggers subsequent Ca^{2+} release from the SR [1,2]. This Ca^{2+}-mediated communication is thought to require a close association between the cell surface and intracellular ionic channels, because the mobility of Ca^{2+} is highly restricted by cytosolic buffering effects. Skeletal muscle E-C coupling probably requires mechanical interaction between the cell surface dihydropyridine receptor/voltage-gated Ca^{2+} channel and ryanodine receptor [3,4]. The proposed mechanical interaction between the channels on different membrane systems requires a close association between the T-tubule and SR. On the other hand, cardiac and skeletal muscle cells contain junctional membrane structures, called the diad and triad, respectively. In the diad the T-tubule and SR are closely associated while in the triad the T-tubule faces the SR membranes on both sides. The channel proteins for the signal conversion during E-C coupling are indeed enriched in these junctional membrane structures [5].

Our recent studies demonstrated that junctophilin (JP) subtypes are involved in the formation of the junctional membrane structure in excitable cells [6–11]. JP is composed of two major domains, a carboxy-terminal hydrophobic segment spanning the ER/SR membrane and the remaining cytoplasmic region interacting with the plasma membrane [6,7]. The functional expression of JP in amphibian embryonic cells thus produces the junctional membrane complex between the plasma membrane and the ER [6]. So far, we have identified three JP subtypes encoded by different genes, namely JP-1, 2 and 3, in mammalian tissues [6,7]. In cardiac muscle bearing the diad, JP-2 is specifically expressed. In JP-2-knockout mice showing embryonic lethality, the mutant cardiac myocytes exhibit deficiency of peripheral coupling, an immature form of junctional membrane structure in striated muscles, and resulting abnormal Ca^{2+} release from the SR [6,8]. In mature skeletal muscle bearing the triad, both JP-1 and JP-2 are expressed, and triad formation during development after birth seems to correlate well with the induction of JP-1 expression [9,10]. The disruption of the JP-1 gene produces deficiency of triad junction in skeletal muscle cells, and JP-1-knockout mice show neonatal lethality probably due to insufficient muscle E-C coupling [9]. These observations likely suggest that JP-1 is a key molecule for the structural conversion of the diad into the triad in skeletal muscle, and also raise the question whether the ectopic expression of JP-1 could induce the structural change of the diad to the triad in cardiac myocytes. In this report we examined the assumption using transgenic mice.

2. Materials and methods

2.1. Generation of transgenic mice

For cardiac muscle-specific expression of JP-1, the α-myosin heavy

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chain (MHC) promoter-containing expression vector [12] was utilized for transgene construction as shown in Fig. 1. A synthetic *Xho*I linker was introduced into the *Sma*I site in the 5′ non-coding region of mouse JP-1 cDNA [6], and the resulting cDNA was digested with *Xho*I and *Hin*dIII to yield the 3.2-kb fragment containing the full-length coding sequence. The cDNA fragment was inserted into the expression plasmid at the cloning site between *Sal*I and *Hin*dIII. After *Not*I digestion, the transgene was purified and injected into pronuclei of eggs from C57BL mice. The injected eggs were transferred into the oviducts of pseudopregnant ICR mice to generate transgenic mice. The integration of the transgene was examined by polymerase chain reaction (PCR) with transgene-specific primers, and two independent lines of transgenic mice were successfully created. Young adult mice (8–10 weeks old) were used in this report. The transgene injection was carried out in a commercial facility (SLC, Japan), and all protocols described in this paper were in accordance with the guidelines of Tohoku University.

2.2. Immunochemical analysis

Antibodies specific to mouse JP-1 and JP-2 were prepared as described previously, and they showed no cross-reactivity to each other [6,9]. Immunoblot analysis was performed essentially as described previously [6]. Briefly, total microsome was prepared from mouse hearts and skeletal muscle, and microsomal proteins were separated on sodium dodecyl sulfate–polyacrylamide gels and transferred to nylon filters. After the reaction of primary and peroxidase-conjugated secondary antibodies, immunoreactivities were visualized using a staining kit (DAB kit, Nakalai Tesque, Japan). For immunohistochemical observations, mouse hearts were fixed in cold acetone, embedded in OCT compound, and quick-frozen in liquid nitrogen. Cryosections (10–20 μm thickness) were reacted with primary and FITC- and rhodamine-conjugated secondary antibodies and examined using a fluorescence microscope (BX60 BX-FLA, Olympus) equipped with a cooled CCD camera (SV-16E, Koheisha, Japan).

2.3. Ultrastructural analysis

Ultrastructural analysis was performed essentially as described previously [6]. Briefly, mouse hearts were fixed in 3% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate buffer (pH 7.4), and were post-fixed in 1% OsO_4 and 0.1 M cacodylate buffer (pH 7.4). After washing with the buffer solution, the tissues were dehydrated using alcohol and acetone, and then embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined under an electron microscope (JEM-200CX, Jeol).

3. Results

3.1. Generation of transgenic mice expressing JP-1 in cardiac myocytes

To examine the effects of JP-1 expression in cardiac muscle, we constructed the transgene as shown in Fig. 1A. In the transgene, JP-1 mRNA is expressed under the control of the α-MHC promoter, and thus heart-specific activation of the gene is expected. Of the mice derived from C57BL eggs injected with the transgene, three were positive in our PCR screening (data not shown). The chromosomal integration of the transgene was further confirmed by Southern blot analysis as shown in Fig. 1B. The mice, numbered 17 and 12, carried high copy numbers of the transgene, and were used to establish transgenic mouse lines. F1 mice were generated by crossing between the breeder and C57BL mice and analyzed in the experiments below. The transgenic mice were apparently normal in health, growth, and reproduction in the heterozygous state under our conventional housing conditions.

Expression of JP subtypes in the heart was examined by Western blot analysis using microsomal preparations (Fig. 2A). In hearts from wild-type mice, JP-2 was abundantly expressed, while JP-1 could not be detected at the protein level. On the other hand, both JP-1 and JP-2 were abundantly expressed in the two lines of transgenic mice due to activation of the transgene. The expression levels of JP-1 were in similar ranges in total microsomes from skeletal muscle of wild-type mice and hearts of the transgenic mice. On the other hand, no significant change in the expression level of JP-2 was observed between the transgenic and wild-type mice. Therefore, the transgenic mice seem to provide an ideal experimental system for analyzing the effects of ectopic expression of JP-1 in cardiac myocytes.

3.2. Subcellular localization of expressed JP-1 in cardiac myocytes

In cardiac muscle, the T-tubule runs alongside the Z-line and associates in parts with the SR membrane to form the diad. Therefore, in immunohistochemical observation of cardiac myocytes, the molecules specifically localized in the diad, for example, the ryanodine and dihydropyridine receptors, show the strip-staining pattern. As reported previously [6], antibody against JP-2 mainly recognizes rows along the Z-line, suggesting the selective localization of JP-2 in the diad. We could not detect a significant difference in the staining pattern with the anti-JP-2 antibody between cardiac myocytes from the transgenic and wild-type mice (Fig. 2B-a and

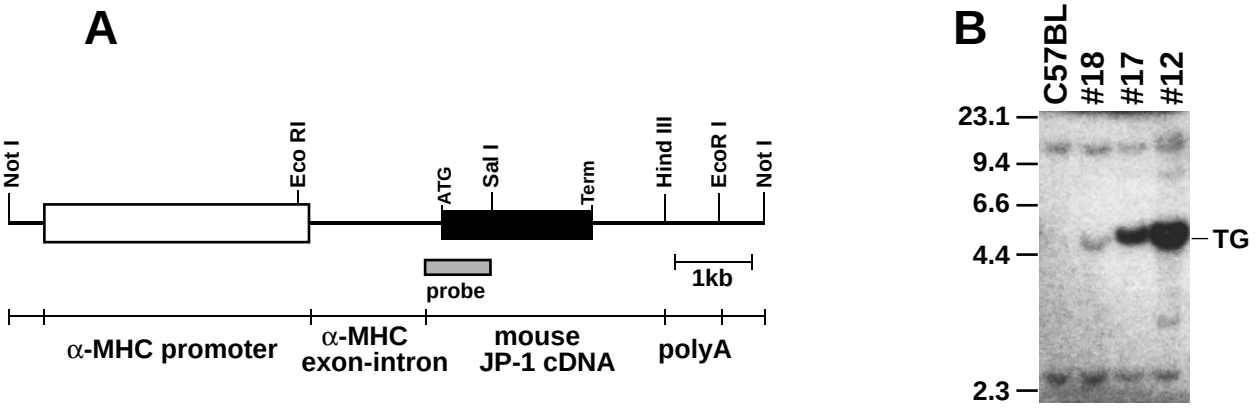

Fig. 1. Generation of mutant mice carrying α-MHC-JP-1 transgene. Structure of the α-MHC–JP-1 transgene (A). The α-MHC promoter and mouse JP-1 cDNA are indicated by open and filled boxes, respectively. Southern blot analysis for α-MHC–JP-1 transgene (B). Genomic DNA preparations were digested with *Eco*RI, and analyzed using the probe shown in A. The hybridization signal derived from the transgene is marked by TG and is not detected in wild-type mice. Signals derived from the endogenous JP-1 gene were observed as ~15- and ~2.5-kb bands. Size markers are in kb.

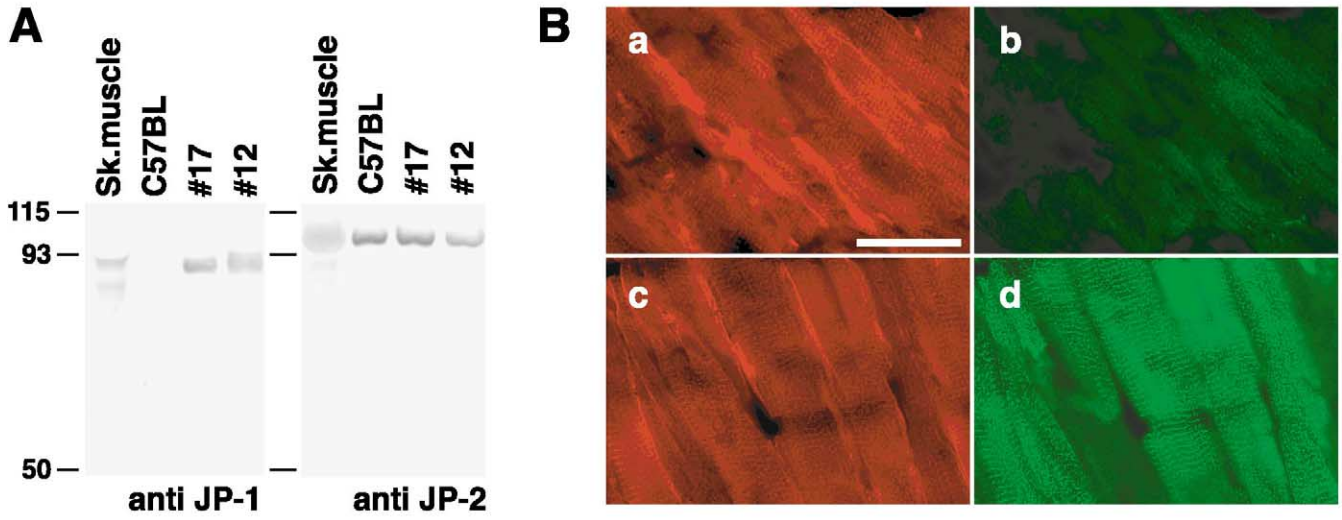

Fig. 2. Expression of JP-1 in cardiac myocytes from transgenic mice. Immunoblot analysis of cardiac microsomal proteins using antibodies against JP-1 and JP-2 (A). Membrane preparations (12 μg protein) were analyzed, and microsome from skeletal muscle expressing both JP-1 and JP-2 was used as a positive control. Ectopic expression of JP-1 in the heart was observed in the transgenic mice. Size markers are in kDa. Immunohistochemical analysis of JP-1 and JP-2 in ventricular myocytes (B). Ventricular myocytes from wild-type (a and b) and #17 transgenic mice (c and d) were double-immunostained with antibodies to JP-2 (a and c) and JP-1 (b and d). Scale bar, 30 μm.

c). Antibody to JP-1 failed to produce signals in the wild-type myocytes (Fig. 2B-b). In the transgenic mice, expression of JP-1 was immunohistochemically detected in most ventricular myocytes (>80%) and a few atrial myocytes (~6%). Both antibodies showed similar staining patterns, and the distribution of JP-1 seemed to overlap with that of JP-2 in the ventricular myocytes (Fig. 2B-c and d). The results obtained suggest that endogenous JP-2 and expressed JP-1 are co-localized in junctional membrane structures in the myocytes from transgenic mice. Because of the absence of histological abnormalities, such as myocyte hypertrophy or fibrosis, in the hearts from the transgenic mice, the ectopic expression of JP-1 seems to cause no obvious morphological and physiological effects at the cellular level.

3.3. *Abnormal junctional membranes in cardiac myocytes expressing JP-1*

To examine the effects of JP-1 on membrane structures, we next examined ventricular myocytes from the transgenic mice under the electron microscope. Most couplings between the T-tubule and SR in wild-type mice were assigned to a typical diad structure, referred to as type 1 coupling in this paper (Figs. 3A and 4A). Additionally, wild-type myocytes sometimes showed couplings in which the SR membranes roll around large portions of the T-tubules, and these structures were classified as type 2 coupling (Figs. 3B and 4A). These normal diads were observed at the region of the Z-band, and the T-tubule united with SR at regular intervals (~12 mm in gap size). In ventricular myocytes from the transgenic mice, most junctional membrane complexes were assigned to type 1 and type 2 couplings, but the ratio of type 2 couplings was markedly higher than that in wild-type myocytes. The transgenic myocytes frequently showed accomplished forms of type 2 coupling, in which almost the entire surroundings of the T-tubules were enclosed by the SR membranes (Fig. 3C). Furthermore, abnormal membrane structures, designated type 3 couplings (Fig. 4A), were observed in myocytes from the transgenic mice; for instance, some SR and T-tubule elements are bonded alternately (Fig. 3D), several SR membranes are closely associated with branched projections of the T-tubule (Fig. 3E), and concentric membrane circles were formed with alternate bonding of the T-tubule and SR (Fig. 3F). Such abnormal structures were formed instead of diads at the Z-band region, and these junctional gaps between the T-tubule and SR membranes were ~12 nm. Fig. 4B shows the results of a statistical comparison of the junctional membrane structures between the transgenic and wild-type mice. Increased formation of type 2 couplings and appearance of abnormal type 3 couplings were shared by both lines of transgenic mice. On the other hand, the ventricular myocytes from the transgenic mice did not show triad structures, which can be typically observed in mature skeletal muscle cells.

4. Discussion

As described in Section 1, previous studies suggest an important role of JP-1 in the morphological maturation of the junctional membrane structure from the diad to the triad in skeletal muscle cells. If the presence or absence of JP-1 in striated muscles is the main determinant of the morphology of junctional membrane complexes, ectopic expression of JP-1 in cardiac myocytes could convert the diad to the triad. The cardiac myocytes from our transgenic mice co-expressing JP-1 and JP-2 contained no authentic triad junctions, and do not support this presumption. Binding partners of JP subtypes on the plasma membrane seem to be shared by amphibian embryonic cells and cultured mammalian cells, and are proposed to be lipid components [6]. On the other hand, the subcellular localization (Fig. 2), together with the biological function demonstrated previously [6,9], suggests that expressed JP-1 contributes to the construction of the junctional membrane complexes in cardiac myocytes from the transgenic mice. Therefore, the present results likely suggest that triad formation absolutely requires as yet unknown skeletal muscle-spe-

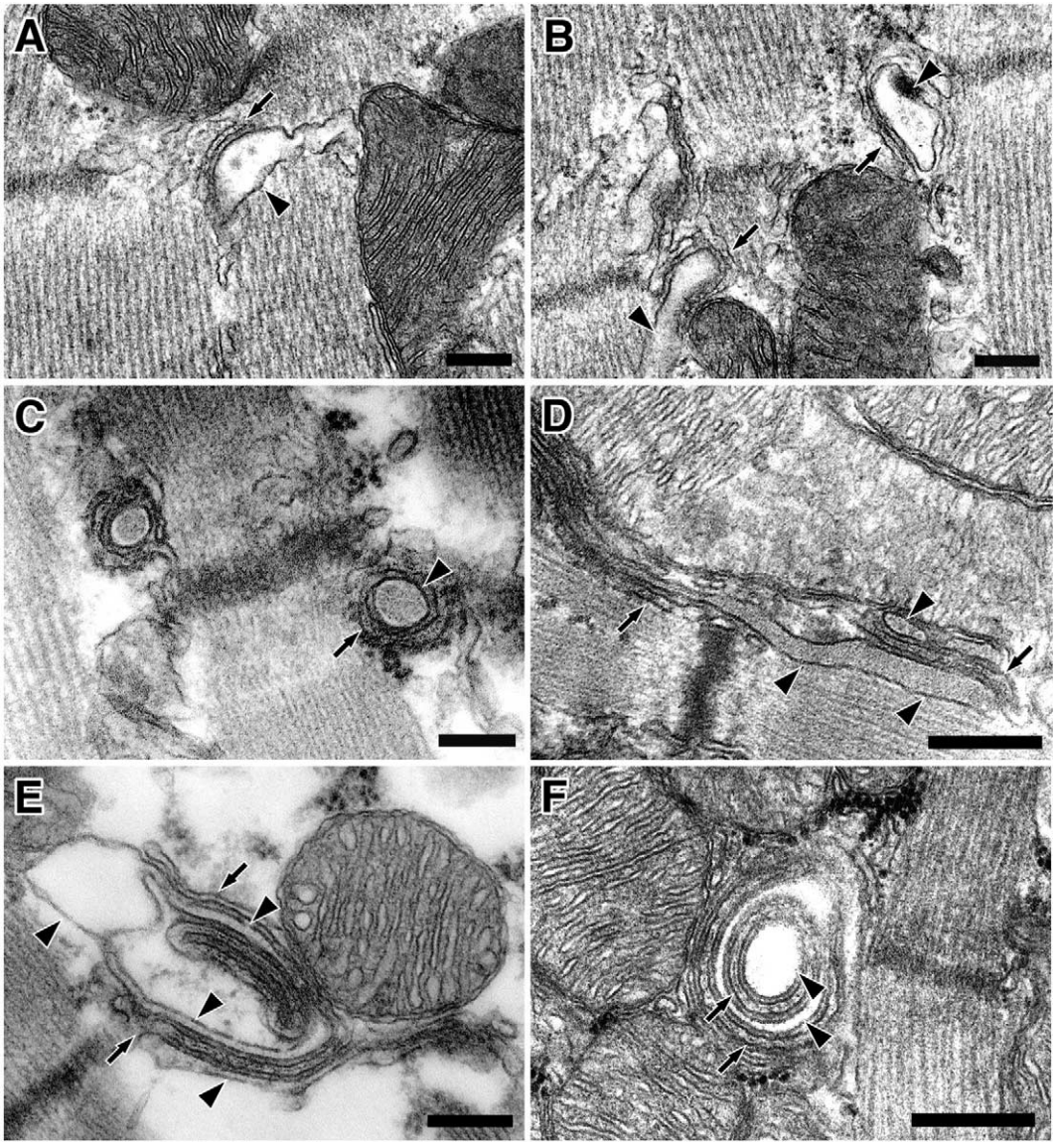

Fig. 3. Ultrastructural analysis of junctional membrane complexes in ventricular myocytes from transgenic mice. Typical diad structures observed in wild-type myocytes are shown in A and B. Irregular junctional membrane structures in myocytes from the transgenic mice are shown in C–F. Arrows and arrowhead indicate the SR and T-tubule, respectively. Scale bars, 0.2 μm in C and 0.1 μm in the others.

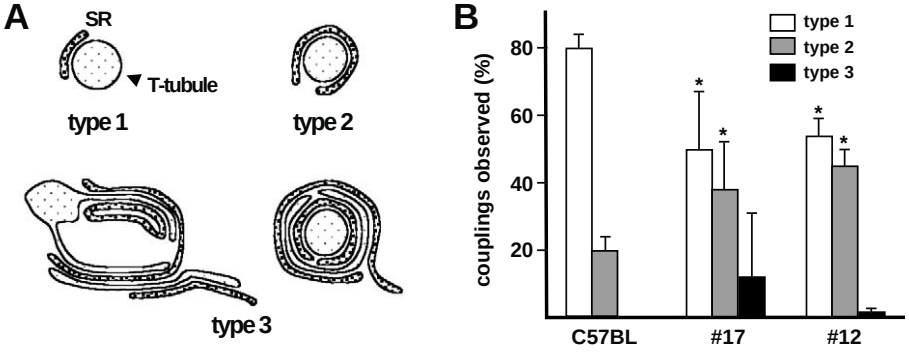

Fig. 4. Statistical analysis of junctional membrane structures in ventricular myocytes from transgenic mice. In this analysis, junctional membrane complexes were classified into three coupling types as in A. The type 3 coupling includes abnormal junctions shown in Fig. 3D–F. A total of 945 junctional membrane structures (on average) from at least three different mice were examined in each group, and data are presented as means ± S.D. in B. Statistical differences between wild-type and transgenic mice are indicated by asterisks ($P < 0.005$ in Student t-test). In two lines of the transgenic mice, formation of type 2 couplings was enhanced, and generation of abnormal type 3 couplings was observed.

cific mechanisms. Because the unknown mechanism is missing, co-expression of JP-1 and JP-2 failed to construct the triad in cardiac myocytes.

Although the triad was not constructed in the cardiac myocytes co-expressing JP-1 and JP-2, formation of the type 2 coupling was facilitated. In contrast, such type 2 couplings have never been observed in skeletal muscle throughout the developmental stages [10]. Again, these observations may suggest that the T-tubule–SR couplings are different between cardiac and skeletal muscles. For instance, there might be certain cellular machinery that prevents excess bonding between T-tubule and SR for arrangement of triad formation in skeletal muscle. In cardiac myocytes from transgenic mice, bonding between the T-tubule and SR might be formed excessively according to the amount of expressed JP-1, and abnormal type 3 couplings might be further developed by irregular association of the T-tubular branches with SR elements.

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