Vertebrate Isoforms of Actin Capping Protein β Have Distinct Functions In Vivo

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Abstract. Actin capping protein (CP) binds barbed ends of actin filaments to regulate actin assembly. CP is an α/β heterodimer. Vertebrates have conserved isoforms of each subunit. Muscle cells contain two β isoforms. β1 is at the Z-line; β2 is at the intercalated disc and cell periphery in general. To investigate the functions of the isoforms, we replaced one isoform with another using expression in hearts of transgenic mice.

Mice expressing β2 had a severe phenotype with juvenile lethality. Myofibril architecture was severely disrupted. The β2 did not localize to the Z-line. Therefore, β1 has a distinct function that includes interactions at the Z-line. Mice expressing β1 showed altered morphology of the intercalated disc, without the lethality or myofibril disruption of the β2-expressing mice.

The in vivo function of CP is presumed to involve binding barbed ends of actin filaments. To test this hypothesis, we expressed a β1 mutant that poorly binds actin. These mice showed both myofibril disruption and intercalated disc remodeling, as predicted.

Therefore, CPβ1 and CPβ2 each have a distinct function that cannot be provided by the other isoform. CPβ1 attaches actin filaments to the Z-line, and CPβ2 organizes the actin at the intercalated discs.

Key words: isoform • heart • cardiomyopathy • Z line • sarcomere

CAPPING protein (CP)[1] is a heterodimer composed of α and β subunits, which binds to the barbed ends of actin filaments. Lower organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, have one gene and one isoform for each of the CP α and β subunits (Amatruda et al., 1990; Waddle et al., 1993; Hopmann et al., 1996). In contrast, vertebrates contain three α subunit isoforms encoded by three different genes, and three β subunit isoforms (β1, β2, and β3) that are produced by alternative splicing from one gene (Schafer et al., 1994; Hart et al., 1997; von Bulow et al., 1997).

The α3 and β3 isoforms are expressed specifically in male germ cells. The β3 isoform is identical to the β2 isoform with the addition of 29 amino acids at the NH_2 terminus (von Bulow et al., 1997). The β1 and β2 isoforms are identical for their first 246 amino acids. Their COOH-terminal ends (β1, 31 residues; β2, 26 residues) are different (Hug et al., 1992; Schafer et al., 1994).

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1. *Abbreviations used in this paper:* α-MyHC, α-myosin heavy chain; CP, capping protein.

Several lines of evidence support the hypothesis that the β1 and β2 isoforms have distinct biochemical and cellular functions that are conserved across vertebrates. First, the COOH-terminal sequences that distinguish the β1 and β2 isoforms are highly conserved across vertebrates (Schafer et al., 1994). Second, the β isoforms display tissue specific expression. The β1 isoform is predominantly expressed in muscle tissue, whereas the β2 isoform is the predominant isoform of nonmuscle tissues (Schafer et al., 1994; Hart et al., 1997). Third, the β isoforms display distinct subcellular localizations within a single cell. In striated muscle cells, β1 localizes to the Z-lines of sarcomeres and β2 localizes to cell–cell junctions and the plasma membrane in general (Schafer et al., 1994).

The presumed functional differences between CPβ1 and CPβ2 that might account for the localization differences and the sequence conservation remain undefined. The COOH-terminal region of CPβ, the region where the isoforms differ, is necessary for binding to actin. However, CPβ1 and CPβ2 bind to actin with essentially identical affinities and kinetics in vitro (Schafer et al., 1996). Also, CPβ1 and CPβ2 are both inhibited by PIP_2, the only known regulator of CP (Schafer et al., 1996).

We wish to understand why vertebrates evolved to have conserved CPβ isoforms that are placed in different subcellular locations in myocytes. One hypothesis is that the β1 and β2 isoforms perform different functions related to

the organization of actin filaments of myocytes. The β1 isoform may have a function that the β2 isoform does not have, β2 may have a function that the β1 does not have, or both may be the case. The first part of this hypothesis predicts that the β2 isoform cannot function in place of β1 in the organization of thin filaments at the Z-line of the sarcomere. Replacement of the β1 isoform with the β2 isoform would cause improper assembly of the actin filaments of the sarcomeres, altering the organization of the sarcomeres and the architecture of the myofibrils. The second part of the hypothesis predicts that the β1 isoform cannot function in place of the β2 isoform in the organization of thin filaments at the intercalated discs. Replacement of the β1 isoform with the β2 isoform would alter actin filament assembly at intercalated discs, leading to altered intercalated disc structure.

An alternative hypothesis is that the β1 and β2 protein isoforms have identical functional abilities within the myocyte, but the alternative transcripts have different regulatory sequences that lead to different expression programs. This hypothesis predicts that the CPβ protein isoforms are functionally interchangeable. In this case, replacement of one protein isoform with the other isoform would not change the actin organization.

To determine if one isoform can function in place of the other in cardiac myocytes, we generated transgenic mice that overexpress CPβ1 or CPβ2 in the ventricles of the murine heart. We used a cardiac-specific promoter, α-myosin heavy chain (α-MyHC), that turns on in the ventricles at birth.

CP is an α/β heterodimer. Single subunits are unstable in vivo and show little or no function in vitro and in vivo, based on results involving mutations, antisense, and expression studies in yeast, *Dictyostelium*, and cultured vertebrate muscle cells and fibroblasts (Amatruda et al., 1992; Hug et al., 1992, 1995; Schafer et al., 1992). Therefore, increasing the expression of one isoform should lead to a replacement of the endogenous isoform in the functional α/β heterodimer.

We found that overexpression of the β2 isoform resulted in severe disruption of myofibril architecture. Overexpression of the β1 isoform resulted in an altered architecture of the intercalated disc. These results indicate that the isoforms do have different functions. Overexpressed CPβ2 does not go to the Z-line; therefore, the unique function of CPβ1 may involve its interaction with components of the Z-line. Likewise, overexpressed CPβ1 does not go to the intercalated disc; therefore, the unique function of CPβ2 may involve its interaction with components of the intercalated disc. Actin binding appears to be necessary for the function of both isoforms in both locations.

Materials and Methods

Construction of the α-MyHC-CPβ1, CPβ2, and CPβ1-L262R Transgene

The coding regions of murine CPβ1 and -β2 (GenBank/EMBL/DDBJ accession no. U10406, U10407) were amplified using PCR with 5′ GCGCGTCGACGCCACCATGAGCGATCAGC 3′ (primer 1) and 5′ GCGCGTCGACAGAGATGGCGCTGCGTGGTC 3′ (primer 2) and inserted downstream of the α-MyHC promoter in a unique SalI restriction site from plasmid clone 26, provided by Dr. J. Robbins (University of Cincinnati; Palermo et al., 1995). A 611-bp HindIII–EcoRI fragment containing the human growth hormone poly(A) signal was at the 3′ end of the α-MyHC/CPβ constructs. A third construct, α-MyHC-β1L262R, which contains a T to G transversion at base 839, changing amino acid 262 from leucine to arginine, was amplified using PCR. Primer 1 and 5′ CAGCACTTGAGAACGTTCCCTCTGCAACTGC 3′ were used to make one product. Primer 2 and 5′ GCAGTTGCAGAGGGAACGTTCTCAAGTGCTG 3′ were used to make another product. The products were then used as template in a final PCR reaction with primers 1 and 2 to generate the full-length product.

The complete open reading frames of the recombinant plasmids were sequenced using specific primers. No changes were present. To prepare the DNA constructs for injection, plasmid containing α-MyHC promoter/cDNA construct was digested with NotI to release the transgene DNA fragment. The fragment was separated from the vector by agarose gel electrophoresis, purified using QiaexII (Qiagen), phenol/chloroform extracted, alcohol precipitated, resuspended, and dialyzed against endotoxin free 5 mM Tris, pH 8.0, 1 mM EDTA. The DNA was quantitated before injection by optical density and agarose gel electrophoresis using a known standard.

Production of Transgenic Mice

The linearized transgene constructs were microinjected into the male pronuclei of one-cell embryos, which were then surgically reimplanted into pseudopregnant female mice. DNA was purified from the tail clips of progeny by digestion at 55°C overnight with 50 mM Tris-HCL, pH 8.0, 100 mM EDTA, 0.5% SDS, and 500 μg/ml proteinase K (Boehringer Mannheim Corp.). DNA was purified using Phase Lock Gel I (Heavy; 5 Prime-3 Prime) and resuspended in 100 μl of 10 mM Tris, pH 8.0, 1 mM EDTA. Progeny were screened for the presence of the transgene by genomic Southern blot analysis using a ^{32}P-labeled 1.2 kb SalI–EcoRI fragment of the α-MyHC promoter. Three different founder lines were identified for α-MyHC-wtβ1, three for α-MyHC-wtβ2, and four for α-MyHC-β1L262R. Founders were bred against a C57BL/6 background and studied as N1 heterozygotes. Founder lines with the greatest expression and most severe phenotypes are described in this paper.

Transgene copy number was quantitated for the progeny of the three founder lines for α-MyHC-wtβ1 by PhosphoImager analysis of genomic Southern blots. The transgene copy numbers for lines 1, 2, and 3 were 2, 4, and 7, respectively. Genomic DNA digested with EcoRI and SalI were probed with a ^{32}P-labeled 1.2-kb SalI–EcoRI fragment of the α-MyHC promoter. The transgene generated a 1.7-kb band, and the endogenous α-MyHC gene generated a 2.5-kb band. The intensities of the radioactivity associated with each band were measured with the PhosphoImager, and the endogenous gene was used as the standard for a single-copy gene. For the α-MyHC-wtβ2 and α-MyHC-β1L262R transgenic lines, genomic Southern blots were evaluated qualitatively and showed transgene copy numbers of 2 to 10, based on the intensity of the transgene band relative to the endogenous gene band.

Determination of Expression Levels

RNA. To evaluate transgene expression by Northern blot analysis, hearts were dissected from 3-wk-old pups, pulverized on dry ice, and homogenized in Trizol reagent (GIBCO BRL). RNA was extracted according to the manufacturer's protocol (GIBCO BRL). Total RNA (10 μg/lane) was size-fractionated in formaldehyde agarose, transferred to Hybond nylon membrane (Amersham Life Science), and hybridized in Rapid-hyb buffer (Amersham Life Science) with a 3′ human growth hormone fragment unique to the transgene. Radiolabeled RNA blots were quantitated with a PhosphoImager (Molecular Dynamics) and each value was normalized to β-actin expression (human β-actin cDNA control probe; CLONTECH).

Protein. Levels of CPβ1, CPβ2, and CPα protein were assayed by immunoblotting using SDS polyacrylamide gel and two dimensional electrophoresis of mouse hearts with isoform specific antibodies as previously described (Schafer et al., 1994). The differences in mass and pI of mouse α1 (32.7 kD, 5.3); α2 (32.9 kD, 5.7); β1 (30.6 kD, 5.5); β2 (31.4 kD, 5.8); and β1-L262R (30.6 kD, 5.8) polypeptides were the basis for their identification. Blots were probed with isoform-specific polyclonal antibodies to β1 (R33) and β2 (R25), a pan β antibody (R22), and an mAb (5B12) that recognizes the α1 and α2 isoforms equally well (Schafer et al., 1994; Hart et al., 1997).

For the SDS polyacrylamide gels, two whole heart extracts from each transgenic line were prepared in SDS sample buffer. Approximately equal amounts of protein were run on 15% SDS-polyacrylamide gels. One gel

was stained with Coomassie blue to confirm equal protein loading for each sample and the other gels were transferred to nitrocellulose and probed. The immunoblots were developed as described (Schafer et al., 1994) and scanned to obtain a digital image. The β1 and β2 protein were quantitated using NIH image and compared with the signal intensities of immunoblots of known concentrations of purified β1 and β2 probed with the same antibody (Schafer et al., 1998). These measurements were normalized to the amount of protein loaded on the gels. For statistical purposes, Western blot analyses of proteins were performed twice with each sample and mean ± values calculated.

Heart/Body Weight Ratios

Transgenic mice and their nontransgenic littermates, from 9–180-d-old, were anesthetized. Their beating hearts were excised, rinsed, drained of fluid, and weighed. Heart/body weight ratios were calculated and expressed as mg:g.

Preparation of Samples for Microscopic Examination

Hearts were excised and fixed overnight in 10% buffered formalin. The hearts were cut in half along a midsagittal plane, paraffin-embedded, and sectioned. Sections were stained with hematoxylin and eosin. For immunofluorescence, sections were deparaffinized with xylene and rehydrated through a series of graded alcohol washes to distilled water. Sections were labeled with antibodies to CPβ1 (R33), CPβ2 (R25), actin (C4, a gift of Dr. J. Lessard, University of Cincinnati), and vinculin (Sigma Immunochemicals). Primary antibodies were detected by fluorescently tagged secondary goat anti–rabbit (Cy3; Jackson ImmunoResearch Laboratories) or goat anti–mouse antibodies (DTAF; Sigma Immunochemicals). Sections were mounted in *N*-propylgallate/glycerol mounting media. Immunofluorescence microscopy was performed on an epifluorescence microscope (IX70; Olympus) with a 1.35 NA 100× UPlanApo objective and U-MWIBA (DTAF) and U-MNG (Cy3) filter sets. Images were collected with a cooled CCD video camera (RC300, Dage-MIT).

For EM, hearts were fixed in 2.5% glutaraldehyde, 100 mM calcium cacodylate overnight. A section of the left ventricular wall parallel to the papillary muscle was removed. This section was postfixed in 1.25% osmium tetraoxide, stained en bloc with 4% uranyl acetate, embedded in Polybed (Polysciences), sectioned, and stained with 4% uranyl acetate/Reynold's lead citrate. Thin sections were examined using a Zeiss 902 electron microscope.

Results

CPβ2 Cannot Functionally Replace CPβ1

To test if CPβ2 can functionally replace CPβ1 in organizing the thin filaments at the Z-lines of the sarcomere, we used the α-MyHC to express the CPβ2 isoform in the mouse myocardium. These transgenic lines are referred to as TG-wtβ2. The well-characterized α-MyHC promoter directs strong and specific expression in the murine ventricles beginning at birth. It is not expressed in smooth or skeletal muscle, or in the ventricular chamber during development (Palermo et al., 1995, 1996; Robbins et al., 1995).

Gross Phenotype

Three TG-wtβ2 founders were identified. None of the founders demonstrated any gross phenotype, reduced viability, or reduced fertility. Backcrossing of the TG-wtβ2 founders produced heterozygous N1 offspring. N1 progeny from two of the founders (2 and 3) showed severe effects. The transgenic mice exhibited stunted growth and an irregular gait beginning at approximately seven days after birth. They expired at 7–26 d after birth (Fig. 1). No peripheral edema was noted, but breathing appeared labored for several days before death. The phenotype was completely penetrant, with 100% of heterozygous N1 transgenic mice from these two founders displaying the described phenotypes (Fig. 1).

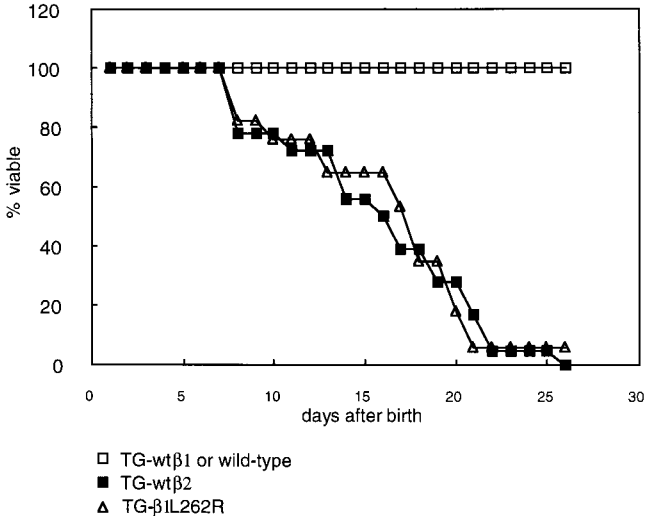

Figure 1. Life span of transgenic mice (wild-type, $n = 66$; TG-wtβ1, $n = 34$; TG-wtβ2, $n = 18$; TG-β1L262R, $n = 17$). The dates of mice found dead or very near death (animals immobile with labored breathing) were recorded and used to determine the life span of the transgenic mice. Each data point indicates the percentage of animals alive at that day after birth. TG-wtβ1 and wild-type mice shared an identical profile, neither having a reduction in viability. In contrast, TG-wtβ2 (lines 2 and 3) and TG-β1L262R (line 2) mice died between 7–26 d after birth.

Because the founders were heterozygous for the transgene insertion, we anticipated 50% of the N1 progeny would be heterozygous for the transgene insertion. However, heterozygous transgenic N1 progeny with the severe phenotype represented only 20–35% of the litter, suggesting that these founders were chimeric for the transgene insertion. In ~20–30% of transgenic mice, the foreign DNA is presumed to integrate at a later stage, resulting in transgenic mice that are mosaic for the transgene (Wilkie et al., 1986). This hypothesis can also account for the fact that the founders had far less severe cardiac phenotypes than the transgenic N1 progeny.

To test whether the phenotype of the TG-wtβ2 mice was due to defective interaction of the actin-based thin filaments with CP at Z-lines, we generated transgenic mice expressing a dominant negative point mutation of CPβ1. These transgenic lines are referred to as TG-β1L262R. This mutation changes amino acid residue 262 from leucine to arginine and lowers the actin-binding affinity by a factor of 10^4 (Barron-Casella et al., 1995). Four founders were identified. None of the founders exhibited any discernable gross phenotype. There was no reduction in viability or fertility.

The phenotype of the N1 TG-β1L262R heterozygotes (TG-β1L262R lines 2 and 3) was similar to that of the N1 TG-wtβ2 heterozygotes. These mice showed stunted growth, an irregular gait, labored breathing, and death at 7–26 d after birth (Fig. 1). This phenotype was almost completely penetrant (16/17 animals). In contrast to the

TG-wtβ2 heterozygotes, one eye often failed to open in the TG-β1L262R heterozygotes, presumably due to α-MyHC's weak expression in the ocular musculature (Sussman et al., 1998).

To determine if the TG-wtβ2 and TG-β1L262R phenotype could be caused by increased expression of CPβ protein in general, we used the same approach to generate transgenic mice that overexpress the CPβ1 isoform. These transgenic lines are referred to as TG-wtβ1. Three founders were identified. Neither the founders, nor the N1 heterozygotes, exhibited any of the gross phenotypes as observed in the TG-wtβ2 transgenic mice (see CPβ1 Cannot Functionally Replace CPβ2).

RNA Expression

To confirm the integrity of the transgenic transcript and to quantify transgene expression, Northern blot analysis using a transgene-specific probe was performed on total RNA from hearts obtained from transgenic and nontransgenic littermates.

Nontransgenic animals showed zero expression of the transgene, as expected. All the TG-wtβ2 transgenic lines showed expression. Line 1 showed the lowest level of expression. Line 2 expression was approximately fourfold that of line 1. Line 3 expression was ~4.5-fold that of line 1 (Fig. 2). N1 heterozygotes from lines 2 and 3 had the previously described strong gross phenotype whereas transgenic line 1 did not. Therefore, transgene transcript accumulation correlated with the severity of the gross phenotype.

Similarly, in the TG-β1-L262R lines, the levels of transgene expression correlated with the severity of the phenotype. A strong heterozygote (line 2) expressed the transgene approximately fourfold more than a weak line (line 1; Fig. 2).

Protein Expression

To determine the level of protein expression of the CPβ1 and CPβ2 isoforms in the hearts of TG-wtβ2, TG-β1L262R, and TG-wtβ1 mice, myocardial protein was quantified by immunoblot with isoform-specific and pan-reactive antibodies.

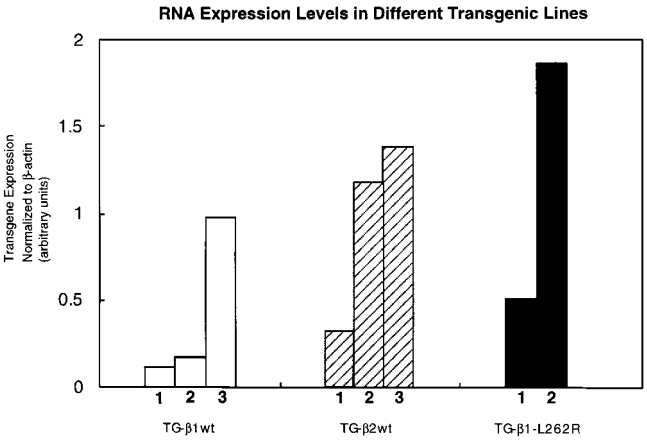

Figure 2. Transgene expression in the TG-wtβ1, TG-wtβ2, and TG-β1L262R founder lines. The lines show different levels of expression, which correlate with the severity of the phenotype. TG-wtβ1, line 3; TG-wtβ2, lines 2 and 3; and TG-β1L262R, line 2 are described further in the paper.

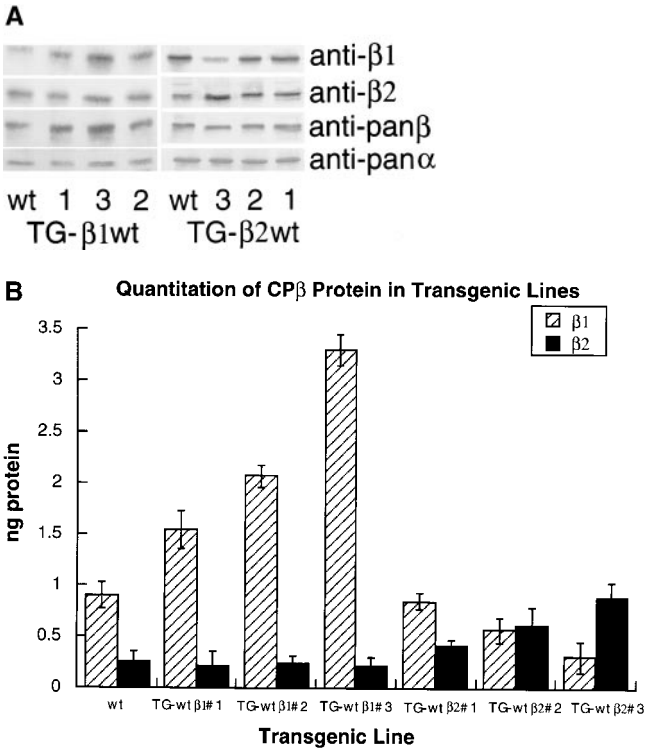

Figure 3. Expression of CPα, CPβ1, and CPβ2 in TG-wtβ1 and TG-wtβ2 lines. A, The expression of the TG-wtβ1 and TG-wtβ2 lines was determined from Western blots probed with anti-β1, anti-β2, anti-panβ, and anti-panα antibodies. B, The intensity of the bands on the blots corresponding to the β1 and β2 proteins was quantitated and compared with the signal intensities of immunoblots of known concentrations of purified β1 and β2 probed with the same antibody. Error bars represent SEM.

TG-wtβ2. For the TG-wtβ2 lines, an increased level of CPβ2 protein was observed in all of the transgenic lines (Fig. 3, anti-β2). The level of CPβ2 protein varied from approximately two- to fourfold that of the wild-type endogenous level. The amount of transgenic protein correlated with the level of transgene transcript, with line 3 containing the highest levels of protein and transcript. Overexpression of β2 led to a decrease in the level of β1 (Fig. 3, anti-β1). The total amount of CPβ and CPα did not change (Fig. 3, anti-panβ, anti-panα). Therefore, the level of active heterodimer was constant, with an increased ratio of α:β2 to α:β1.

TG-β1L262R. To determine the CPβ isoform protein expression in TG-β1L262R lines, two-dimensional immunoblots were required to identify the β isoforms (Fig. 4). Although the β1 and β1-L262R polypeptides have similar molecular masses, the β1-L262R mutation shifts the pI from 5.5 to 5.8 and then can be discriminated from wild-type (see Fig. 4, cartoon). The blots were also probed with an mAb pan-reactive to α1 and α2.

An increased level of the transgenic protein was observed in hearts of TG-β1L262R animals that have both a

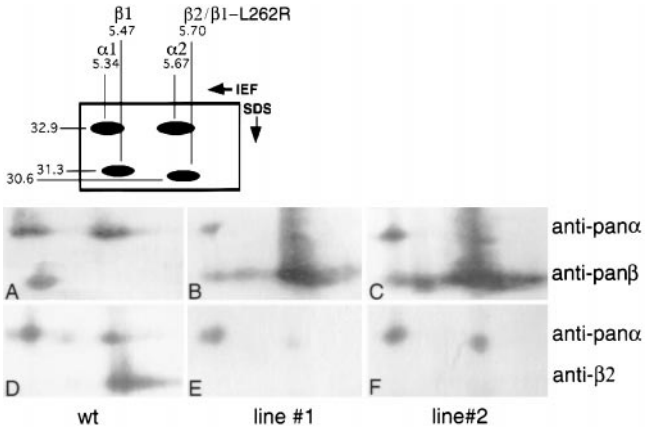

Figure 4. The expression of CPβ1, CPβ1-L262R, CPβ2, and CPα in TG-β1L262R lines. The expression of TG-β1L262R lines was determined by two-dimensional immunoblots probed with anti-panα, anti-β1, and anti-β2. The β1 and β1-L262R polypeptides have a similar molecular mass, but different pI's, 5.5 and 5.8, respectively.

strong (line 2) and a mild (line 1) phenotype (Fig. 4, anti-panβ). The severity of the phenotype correlated with the amount of protein expressed. The increased expression of the β1-L262R polypeptide was accompanied by a decreased level of the endogenous β1 subunit (Fig. 4, B and C) and a complete loss of the β2 subunit (Fig. 4, E and F). There was no change in the level of the CPα subunit expression. Therefore, overexpression of the β1-L262R polypeptide changed the ratio of the isoforms in the heterodimer population with an increase of α:β1-L262R and a decrease in α:β1 and α:β2.

TG-wtβ1. For the TG-wtβ1 lines, an increased level of the CPβ1 protein was seen in all the transgenic lines (Fig. 3). The increased level of CPβ1 protein in the TG-wtβ1 lines was similar to the increased level of CPβ2 protein seen in the TG-wtβ2 lines, when considered as a ratio with respect to wild-type, and was substantially increased when considered in absolute terms. The TG-wtβ1 lines did not show the severe phenotypes of sarcomere disruption and hypertrophic cardiomyopathy seen in the TG-wtβ2 lines (described in detail in CPβ1 Cannot Functionally Replace CPβ2). Therefore, the severe phenotype of the TG-wtβ2 mice was specifically caused by the increased level of CPβ2 protein, and could not be explained by an increased level of β subunit protein in general.

Morphology of the Heart

Gross Morphology. To determine if overexpression of CPβ2 and CPβ1-L262R in the murine myocardium leads to cardiac hypertrophy, cardiac weights corrected for body weight were determined for the TG-wtβ1 and TG-β1L262R mice (Table I). TG-wtβ2 and TG-β1L262R mice had elevated heart to body weight ratios relative to control littermates, ~30% larger than ratios for nontransgenic mice. Therefore, overexpression of CPβ2 and CPβ1-L262R caused cardiac hypertrophy.

Light microscopic analysis of hematoxylin- and eosin-stained heart sections of TG-wtβ2 (lines 2 and 3) and TG-β1L262R (lines 2 and 3) revealed global dilation of the hearts of transgenic, compared with nontransgenic controls (Fig. 5 A). The walls of the ventricles were thickened throughout the chambers. The chambers were not grossly dilated.

Table I. Cardiac Hypertrophy Based on Heart Weight

	Heart/body wt	*n*
	mg/g	
Wild-type	6.8 ± 0.3	19
TG-wtβ1	6.0 ± 0.4	7
TG-wtβ2	9.8 ± 0.8	7
TG-β1L262R	10.6 ± 1.0	9

Heart to body wt ratios are listed as mean ± SEM. *n* is the number of animals.

Ultrastructure of the Myocardium. To determine if cardiac hypertrophy was accompanied by an altered morphology of myofibrils and sarcomere structure, the ventricular walls of control animals (nontransgenic littermates) and transgenic mice were examined by light microscopy and thin section EM.

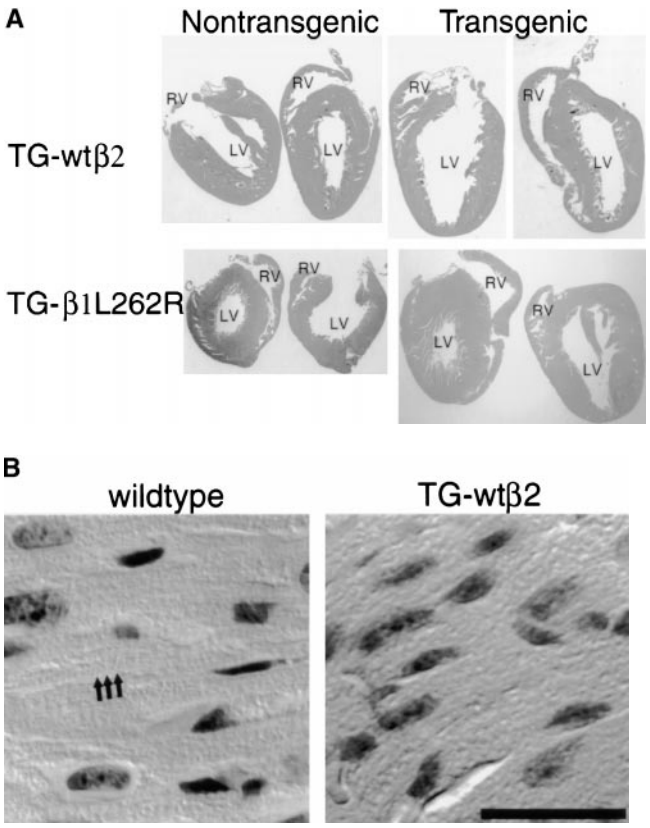

Figure 5. Transgenic mouse hearts exhibit histopathological features of concentric hypertrophy. A, Hematoxylin- and eosin-stained sections show an enlarged heart with thickened left ventricles relative to nontransgenic littermates. B, At higher magnification, the walls of wild-type mice show striations (arrows) reflecting well-ordered periodicity and alignment of myofibrils. The walls from the TG-wtβ2 mice (lines 2 and 3) show no periodicity. Nuclei are enlarged in the TG-wtβ2 samples as well. Bar, 5 μm.

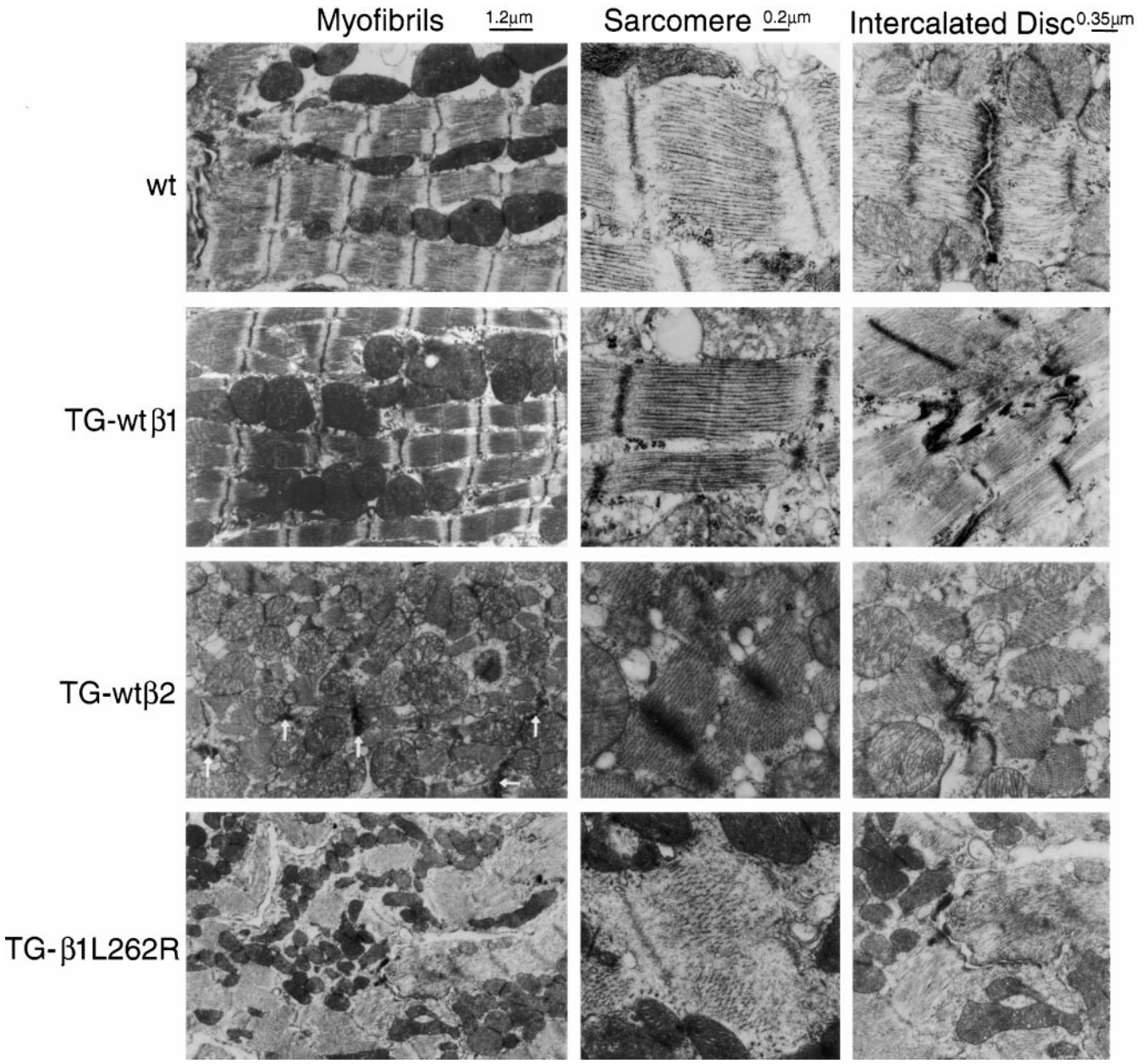

Figure 6. Electron micrographs of thin sections of the left ventricles of control, TG-wtβ1, TG-wtβ2, and TG-β1L262R animals. Myofibrils of controls are well-organized with periodicity along their length and lateral alignment. The myofibrils of TG-wtβ2 (lines 2 and 3) and TG-β1L262R (line 2) were poorly organized, with short or absent Z-lines. The intercalated discs of controls consist of closely opposed, transversely oriented plasma membranes. The intercalated discs of TG-wtβ1 and TG-β1L262R mice showed a loss of organization.

Light microscopic analysis of ventricular walls of nontransgenic mice showed the characteristic pattern of striations. The striations are observed due to the periodicity of the repeating sarcomere units and because the myofibrils are aligned with each other laterally. In contrast, the ventricle wall of the TG-wtβ2 hearts revealed loss of the striations. Also, the size of the myocyte nuclei was increased (Fig. 5 B).

In thin section EM, the hearts from wild-type animals had myofibrils aligned laterally from Z-line to Z-line, with distinct A and I bands, and Z- and M-lines (Fig. 6). Mitochondria had an elongated shape and were between the parallel arrays of myofibrils.

In TG-wtβ2 mice, gross myofibrillar disarray was apparent. The myocytes were arranged in chaotic patterns at oblique and perpendicular angles. In the sarcomere, the I band was difficult to identify. The A band appeared to span the entire length between highly disorganized Z-lines. The Z-lines were truncated, wavy, and appeared slightly thickened. In some areas, the Z-lines were lacking entirely. The myofibrils were filled with numerous swollen mitochondria.

The intercalated discs of the TG-wtβ2 myocardium had a relatively normal ultrastructure, but were increased in number and decreased in length. To confirm this result, intercalated discs were examined by light microscopy using

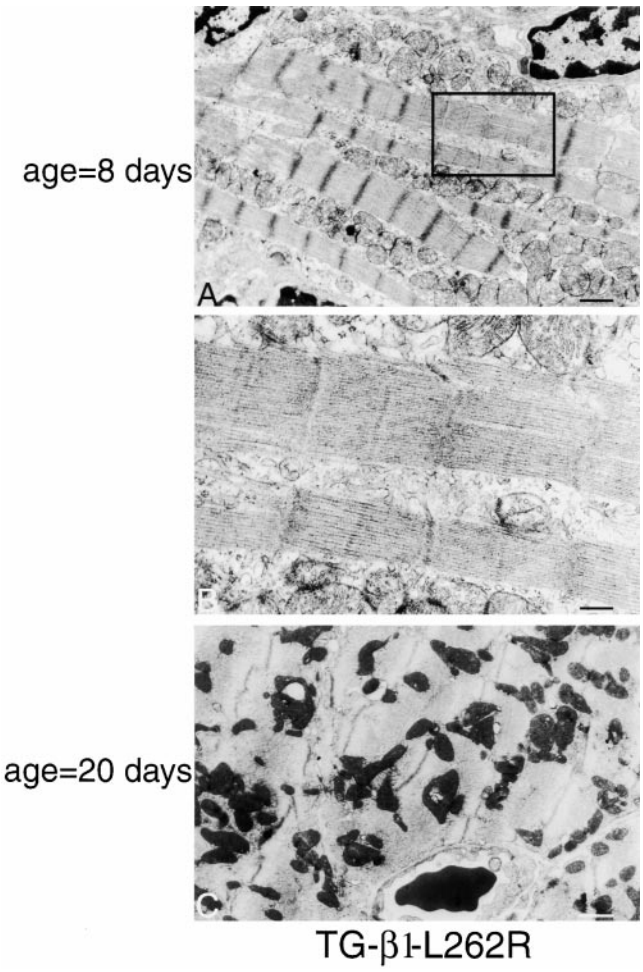

Figure 7. Progressive disorganization. Myofibrils from TG-β1L262R mice (line 2) show progressive disorganization, with milder defects at early times (A and B higher magnification) and poor myofibrillar organization by day 20 (C). Bars, (A and C) 1.1 μm; (B) 0.35 μm.

antivinculin to show the intercalated discs. In normal myocardium, antivinculin staining was found as a fine line at the intercalated discs and weakly at the border of the myocytes (see Fig. 11). In TG-wtβ2 myocardium, vinculin labeling appeared as small irregular lines consistent with the multiplicity of intercalated discs in ultrastructure analysis. This could be due to the addition of new intercalated discs or fragmentation of existing discs caused by the underlying myofibril dysgenesis (see Discussion).

Abnormal cardiomyocyte structure and organization was also apparent in the left ventricles of TG-β1L262R mice, whose mutation precludes the binding of actin to CP (Figs. 6 and 7). This suggests that the myofibrillar disarray observed in the hearts of the TG-wtβ2 mice was due to the inability of CPβ2 to attach the actin filaments to the Z-line. The myofibrils of TG-β1L262R had a highly disorganized architecture, including abnormally registered sarcomeres with disoriented or missing Z-lines. The A and I bands were apparent, but lacked their typical striation. The mitochondria had lost their organization and shape.

Analysis of the myocardium of eight-day-old TG-β1L262R mice showed milder defects, confirming that the myocyte deterioration was progressive from birth as expected (Fig. 7). At eight days after birth, the periodicity of the Z-lines, the registration of the Z-lines, and the alignment of the myofibrils were only slightly altered. Higher magnification showed minor deterioration of the sarcomeres with thinning of the Z-line.

CPβ Protein Localization in Transgenic Hearts

The distribution of the CPβ1 and CPβ2 isoforms in murine heart tissue is the same as that described previously for chicken heart tissue and cultured cardiomyocytes (Schafer et al., 1994). CPβ1 is at the Z-lines of the sarcomere (Fig. 8 A). CPβ2 is at intercalated discs and in a punctate pattern (Fig. 8 C).

TG-wtβ2. Two alternative hypotheses can explain the inability of CPβ2 to functionally replace CPβ1 and organize the thin filaments at the Z-line in the TG-wtβ2 mice. The first is that β2 may not localize to the Z-line due to a lack of targeting information. The second is that β2 may localize to the Z-line, but not bind to and correctly orient the thin filaments there. To discriminate between these alternatives, the localization of CPβ2 in TG-wtβ2 hearts was determined.

In TG-wtβ2 hearts, CPβ2 localized to the periphery of the myofibrils and in a punctate pattern (Fig. 9). Because the Z-lines were truncated and wavy, we used double immunofluorescence labeling with antiactin to identify the Z-line (Fig. 10). Double immunofluorescence labeling with anti-β2 revealed that CPβ2 did not localize to the Z-line in the TG-wtβ2 myocardium. This suggests that CPβ2 is unable to functionally replace β1 at the Z-line due to its inability to localize to the Z-line.

Western blot analysis showed a decreased level of β1 protein in the TG-wtβ2 hearts. To confirm this result and

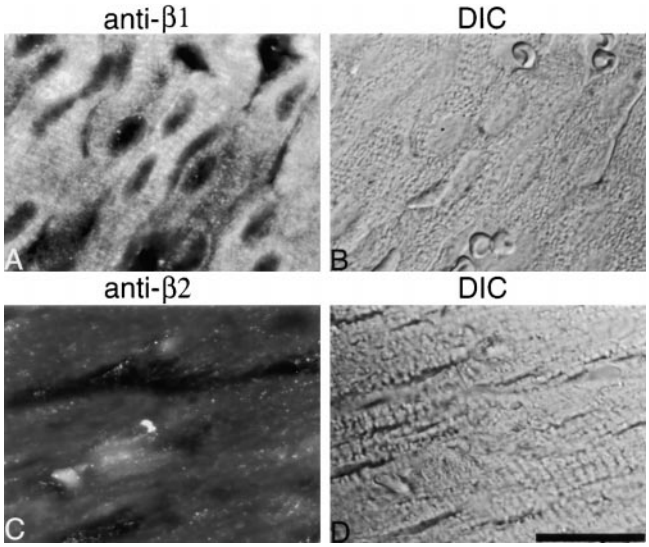

Figure 8. Immunofluorescence staining of the CPβ isoforms (A and C) and corresponding DIC images (B and D) in sections of wild-type hearts. The β1 isoform localizes predominantly to the Z-lines (A). The β2 isoform localizes to intercalated discs and in a punctate pattern at cell–cell junctions (C). Bar, 5 μm.

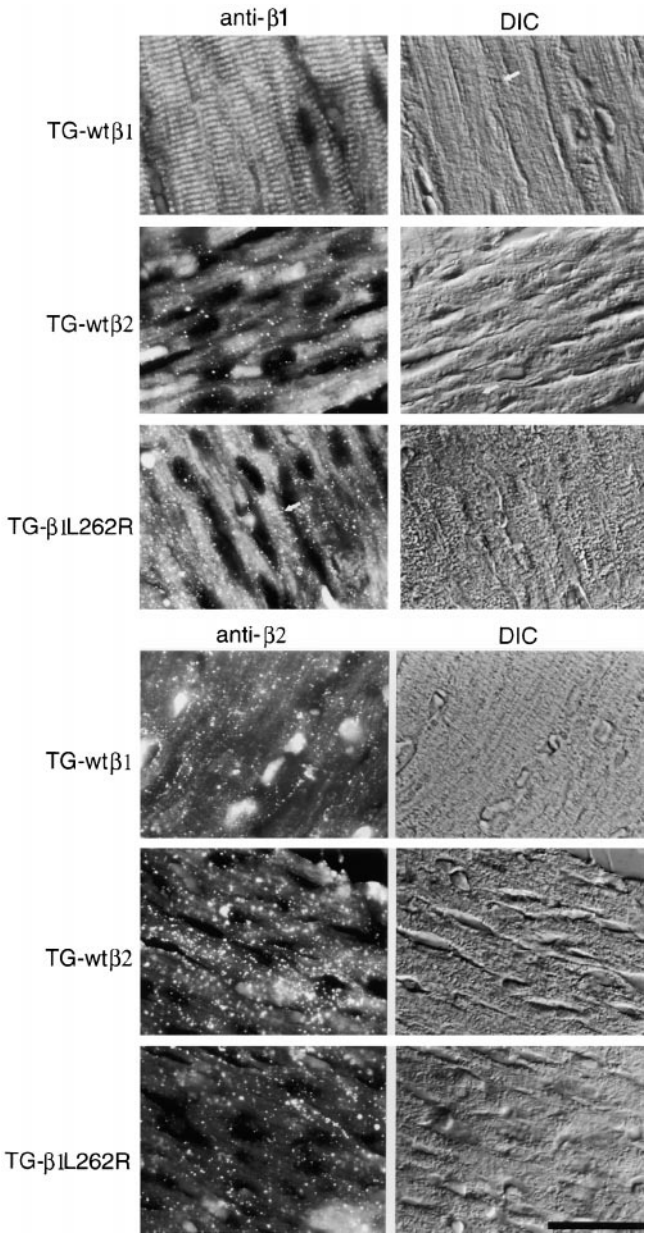

Figure 9. Immunofluorescence staining of CPβ1 and CPβ2 isoforms and corresponding DIC images in TG-wtβ1 (line 3), TG-wtβ2 (line 2), and TG-β1L262R (line 3) hearts. In TG-wtβ1 myocardium, CPβ1 localized to the Z-lines, in a pattern that appeared slightly wider than wild-type. CPβ1 staining intensity was not increased at the intercalated disc (arrow), relative to Z lines. CPβ2 localized to cell–cell junctions, the cell cortex, and in a punctate pattern. In TG-wtβ2 myocardium, CPβ1 localized to incomplete Z-lines; CPβ2 localized to the cell cortex and in a punctate pattern. In TG-β1L262R hearts, CPβ1L-262R protein localized to wavy, fragmented Z-lines (arrows) and in a punctate pattern. Bar, 5 μm.

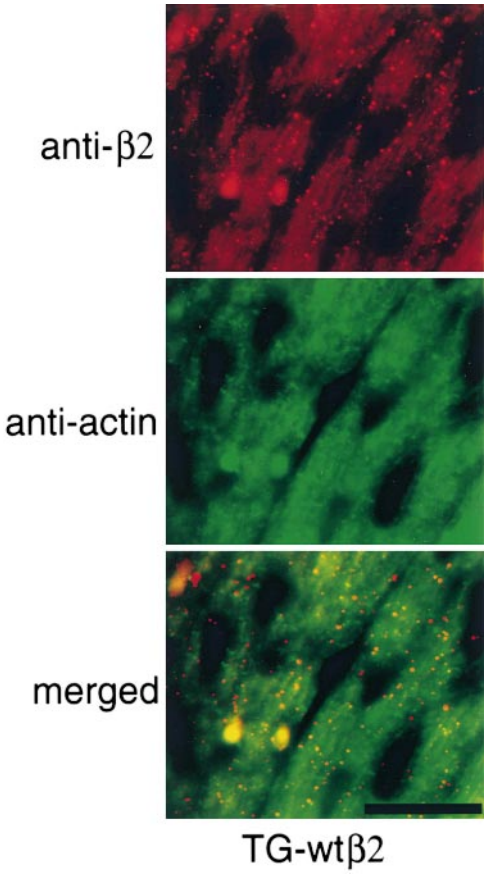

Figure 10. Double immunofluorescence localization of actin and CPβ2 in TG-wtβ2 hearts. Antiactin binds the Z-lines. In TG-wtβ2 (lines 2 and 3) hearts, actin localization at the Z-line was wavy and discontinuous, reflecting the decreased organization of the myofibrils and sarcomeres. β2 localized in a punctate pattern. The merged image shows that β2 does not colocalize with actin at the Z-line in TG-wtβ2 hearts. Bar, 5 μm.

determine the localization of β1 in the TG-wtβ2 hearts, we examined the localization of the β1 isoform with anti-β1. In TG-wtβ2 hearts, β1 was observed as weakly stained truncated Z-lines and in a punctate pattern. The weak level of staining was consistent with the decreased level of β1 seen in Western blot analysis. The truncation of Z-lines was consistent with the myofibril dysgenesis observed in thin section EM (Fig. 9).

TG-β1L262R. β1-L262R is a mutant form of the β1 subunit that binds actin poorly in vitro. We hypothesized that in TG-β1L262R hearts, the mutant β1 protein would localize to the Z-line, but be incapable of binding actin filaments, as expected from its biochemical properties. To determine if β1-L262R protein was able to localize to the Z-lines in transgenic hearts, we examined the localization of β1-L262R with anti-β1. β1-L262R protein did localize to the Z-line, appearing as short, wavy lines, reflecting the loss of the parallel alignment of the Z-lines seen by EM (Fig. 9).

To confirm that the level of β2 protein was reduced in TG-β1L262R hearts, as seen in Western blot analysis, and to examine the localization of β2, we stained heart sections with anti-β2. The intensity of β2 staining was decreased, as expected. β2 localized in a punctate pattern and at cell–cell junctions.

CPβ1 Cannot Functionally Replace CPβ2

We found that CPβ2 could not functionally replace CPβ1

in organizing the actin filaments at the Z-line. We next asked the converse question, whether CPβ1 could functionally replace CPβ2 in organizing the actin cytoskeleton–membrane interactions at intercalated discs. To test this hypothesis, we expressed the CPβ1 isoform in the mouse myocardium using the α-MyHC promoter. Transgenic lines are referred to as TG-wtβ1. Three founders were identified. Neither the founders nor the N1 heterozygotes exhibited any gross phenotype or reduced viability (Fig. 1).

RNA Expression

All of the transgenic TG-wtβ1 lines showed expression (Fig. 2). TG-wtβ1 line 1 had the lowest level. Expression in line 2 was twofold that of line 1, and expression in line 3 was tenfold that of line 1. Nontransgenic littermates showed no expression.

Protein Expression

An increased level of CPβ1 protein was observed in all of the TG-wtβ1 lines, from about two- to fourfold that of endogenous CPβ1 in nontransgenic littermates (Fig. 3). The level of transgenic protein correlated with the level of transgene transcript. Line 3 expressed the highest levels of protein and transcript.

Overexpression of CPβ1 led to an increase in the total amount of CPβ subunit expressed (Fig 4, anti-panβ). The level of the β2 subunit did not change. Importantly, the level of the α1 and α2 subunits did not change. Therefore, the level of active heterodimer was constant, with an increased ratio of α:β1 to α:β2.

Morphology of the Heart

Gross Morphology. To determine if overexpression of CPβ1 in the murine myocardium caused cardiac hypertrophy, heart weights were measured and corrected for body weight (Table I). TG-wtβ1 mice did not have elevated heart to body weight ratios relative to control littermates. Therefore, in contrast to overexpression of CPβ2 and CPβ1-L262R (see above), overexpression of CPβ1 did not cause cardiac hypertrophy.

Light microscopic analysis of hematoxylin- and eosin-stained heart sections of the CPβ1 transgenic mice showed a normal structure of the heart with no enlargement of ventricular walls or chambers (data not shown).

Ultrastructure of the Myocardium. To determine if there was an altered morphology of intercalated discs or myofibrils in the TG-wtβ1 hearts, ventricular walls were examined by thin section EM. In TG-wtβ1 myocardium, the intercalated discs were fragmented, composed of short, disjointed segments that were misaligned relative to the orientation of the myofibrils (Fig. 6). The density of the electron-dense segment parallel to the plasma membrane was diminished. The segment was also discontinuous along its length.

The registration and morphology of the sarcomeres of the TG-wtβ1 myocardium were largely unremarkable (Fig. 6). The Z-lines of the sarcomere were more electron dense and slightly thickened, which may be caused by the increased level of CPβ1 protein seen in Western blot analysis (Fig. 3).

Since this phenotype was relatively mild compared with expression of β2, we considered that the β1 isoform might be functioning at the intercalated disc. To address this question, we examined the intercalated discs of transgenic mice expressing a mutant form of the β1 subunit, TG-β1L262R, that should not function because it binds actin poorly in vitro. The TG-β1L262R mice showed intercalated disc remodeling similar to that of the mice expressing the wild-type β1 isoform, with fragmentation of the intercalated disc and a reduction of electron-dense material parallel to the plasma membrane. The intercalated discs were also examined by light microscopy using antivinculin to show the intercalated discs. In TG-wtβ1 and TG-β1L262R myocardium, vinculin labeling at the intercalated discs appeared as slightly thickened, skewed lines, consistent with the misalignment of the intercalated discs seen in ultrastructure analysis (Fig. 11). Therefore, the wild-type β1 isoform appears not to function at all in place of the β2 isoform at the intercalated disc.

CPβ1 Does Not Accumulate at the Intercalated Discs of TG-wtβ1 Hearts

Two alternative hypotheses can explain the inability of CPβ1 to functionally replace CPβ2 and organize the thin filaments at the intercalated discs in TG-wtβ1 mice. The first is that β1 may not localize to the intercalated disc. The second is that β1 may localize to the intercalated disc, but not bind to and correctly orient the thin filaments there. To discriminate between these alternatives, the localization of CPβ1 in TG-wtβ1 hearts was determined.

Intercalated discs do contain Z-lines from each of the adjoining myocytes. CPβ1 is present at the intercalated disc for that reason, but its staining intensity is not increased relative to other Z-lines in the myofibril. In contrast, CPβ2 is seen at the intercalated discs, but not at Z-lines (Schafer et al., 1994; Fig. 8).

In TG-wtβ1 myocardium, β1 localized to the Z-lines of the sarcomeres, including the Z-line component of the intercalated discs, in a slightly broader pattern than in wild-type (Fig. 9). However, β1 staining intensity was not increased at the intercalated discs. This result suggests that CPβ1 is unable to functionally replace β2 at the intercalated disc due to its inability to localize to the intercalated disc.

Discussion

The CPβ Isoforms Are Functionally Distinct in the Murine Myocardium

CP, an α/β heterodimer, is a component of the actin cytoskeleton in all eukaryotes (Schafer et al., 1994; Hopmann et al., 1996; Hart et al., 1997). Vertebrates contain three α subunit isoforms (α1, α2, and α3) encoded by three different genes and three β subunit isoforms (β1, β2, and β3), which are produced from one gene by alternative splicing.

The sequences of the β1 and β2 isoforms are highly conserved across vertebrates and the β1 and β2 isoforms show different expression and localization patterns (Schafer et al., 1994). In myocytes, β1 is a component of the Z-lines of

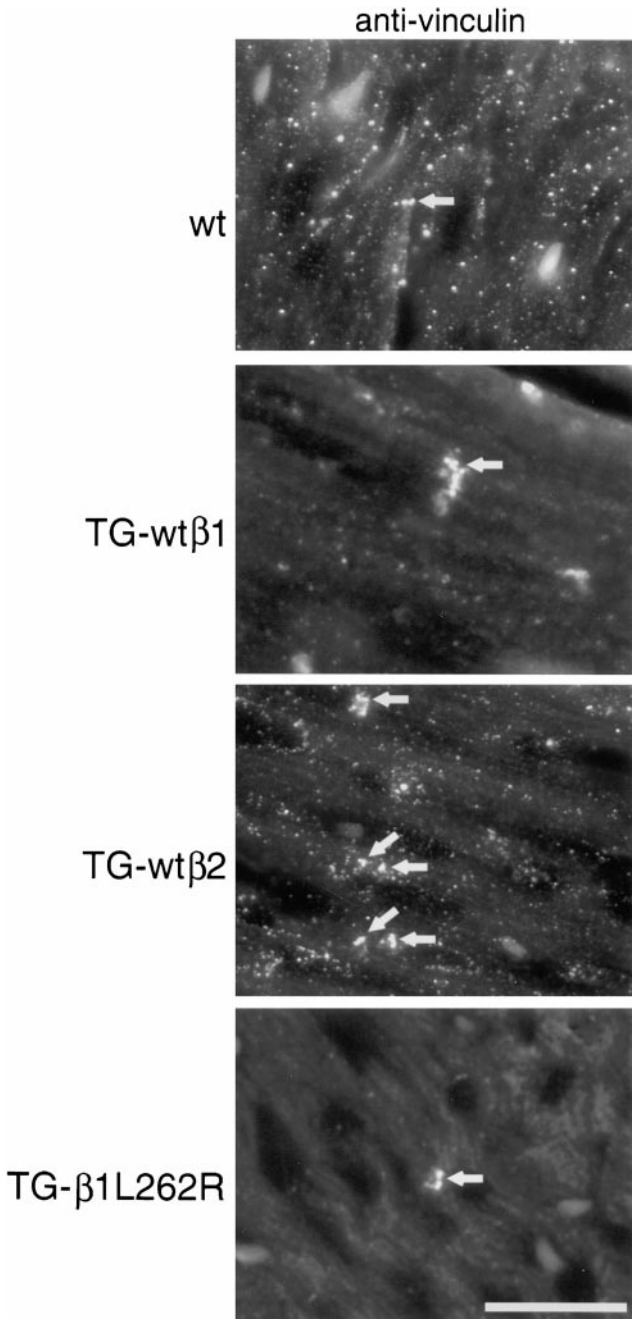

Figure 11. Immunofluorescence localization of vinculin in wild-type, TG-wtβ1 (line 3), TG-wtβ2 (lines 2 and 3), and TG-β1L262R (line 3) hearts. In wild-type myocardium, vinculin localized to a line at the intercalated discs (arrow) and weakly at the border of the myocytes. In TG-wtβ1 and TG-β1L262R hearts, the localization of vinculin to the intercalated disc was a curved line (arrow) that was not transverse relative to the orientation of the myofibrils. In TG-wtβ2 hearts, vinculin localized to intercalated discs in small truncated lines (arrows). Bar, 5 μm.

sarcomeres and β2 is a component of cell–cell junctions, where myofibrils associate with the plasma membrane.

We considered two hypotheses to explain why the β1 and β2 isoforms have conserved sequence differences and unique expression patterns in myocytes. One hypothesis is that the β1 and β2 isoforms are functionally equivalent, but have acquired different expression patterns important for muscle differentiation. This hypothesis predicts that the β1 isoform can replace the function of the β2 isoform and that the β2 isoform can replace the function of the β1 isoform. To test this hypothesis, we shifted the β1:β2 isoform ratio in the mouse heart using expression from a cardiac specific promoter. Our findings exclude this hypothesis because differences in the β1:β2 isoform ratio changed the morphology and physiology of the heart.

Another hypothesis is that the β isoforms have acquired novel biochemical functions and fulfill different roles within the heart. This hypothesis predicts that an increase in the β2 isoform will lead to an alteration in the structure and function of the sarcomeres, which contain β1, and that an increase in β1 will lead to an alteration in the structure and function of the intercalated discs, which contain β2. Our findings support this hypothesis. We found that β2 cannot replace β1 at the Z-line. Expression of wild-type β2 subunit or an actin-binding mutant form of β1 caused major structural defects in sarcomere organization, leading to cardiac hypertrophy and juvenile lethality. We also found that β1 cannot replace β2 at the intercalated disc. Expression of wild-type β1 subunit or an actin-binding mutant form of β1 caused structural defects in the intercalated discs.

Overexpression of CPβ2 or an Actin-Binding Mutant of CPβ1 Causes Hypertrophic Cardiomyopathy

In striated muscle, interactions among actin thin filaments and various contractile proteins contribute to the highly ordered structure and function of muscle. The actin thin filaments of the sarcomere are attached to Z-lines. CP binds the barbed ends of the thin filaments at the Z-line, maintaining the actin filament location, polarity, and length.

We found that inhibiting CP's ability to attach the barbed end of an actin filament to the Z-line has severe effects on sarcomere assembly in mice, consistent with the effects seen in cultured cells (Schafer et al., 1995). Transgenic mice that overexpress the β2 isoform or an actin-binding mutant of the β1 isoform showed juvenile lethality with severe disruption of myofibril architecture and cardiac hypertrophy. This result argues that the isoform-specific function of CPβ1 is to maintain the organization of the sarcomere by attachment of thin filaments to the Z-line. Presumably, the loss of β1 function leads to a loss in the number of thin filaments that can be attached to a Z-line, leading to myofibrillar disarray.

The overexpressed wild-type β2 subunit did not localize to the Z-line. This suggests that the unique function of the β1 isoform involves interacting with additional components of the Z-line, not only actin. This conclusion is also supported by previous work on cultured cells, where an anti-CPβ1 antibody that prevents the binding of CP to actin was observed to localize to the Z-line (Schafer et al., 1995). Together, these results indicate that CPβ1 binds Z-lines independent of actin, by some additional interaction. The components responsible for the isoform-specific interaction remain to be defined. Potential candidates include other sarcomere components present at the Z-line,

such as titin (Pierobon et al., 1989; Soteriou et al., 1993), α-actinin (Papa et al., 1999), and nebulin (Keller, 1995).

Our studies define CPβ1 as a sarcomeric protein for which mutation can cause hypertrophic cardiomyopathy, a disorder characterized by increased left ventricular mass and myofibrillar disarray. Familial hypertrophic cardiomyopathy in humans is caused by mutations in genes encoding sarcomeric proteins, including α-cardiac actin (Mogensen et al., 1999), β-myosin heavy chain (Geisterfer-Lowrance et al., 1990; Tanigawa et al., 1990), troponin T, α tropomyosin (Thierfelder et al., 1994), myosin-binding protein C (Bonne et al., 1995; Watkins et al., 1995b), myosin essential light chain, myosin regulatory light chain (Poetter et al., 1996), and troponin 1 (Kimura et al., 1997). In addition, dilated cardiomyopathy can be caused by mutations in actin (Olson et al., 1998). These actin mutations are hypothesized to affect attachment of thin filaments to the Z-line. The relationships between cardiac hypertrophy and dilatation, and the mechanisms that lead to each of these responses, are being explored in mouse models and humans with cardiomyopathies (Chien, 1999). Using transgenesis, we have generated a new mouse model displaying hypertrophic cardiomyopathy, based on defective thin filament/Z-line attachment. Mutations in CP genes, therefore, merit consideration as causes of human cardiomyopathies.

Overexpression of CPβ1 and an Actin-Binding Mutant of β1 Isoform Causes Altered Intercalated Disc Morphology

Ventricular cardiac muscle cells of the myocardium are connected to one another at their ends by the intercalated disc. The intercalated disc links the Z-lines of adjacent myofibrils and the plasma membranes of adjacent myocytes to one another. The intercalated disc ensures that upon contraction, mechanical tension is efficiently transmitted through the myocardium (Watkins et al., 1995a).

We found that overexpression of CPβ1 or an actin-binding mutant form of CPβ1 caused changes in the structure of the intercalated disc in the myocardium. The intercalated discs of TG-wtβ1 and TG-β1L262R hearts were disjointed and misaligned relative to the orientation of the myofibrils. Presumably, the altered discs were weakened by the loss of CPβ2 and then fragmented in response to contraction.

Multiplicity of Intercalated Discs in TG-wtβ2 Myocardium

Overexpression of CPβ2 led to the formation of multiple truncated intercalated discs in the myocardium; the morphology of the intercalated discs was otherwise normal. The increased number of intercalated discs could be a secondary effect of the underlying myofibril dysgenesis or to increased stress that accompanies hypertrophy (Sepp et al., 1996). However, multiple truncated intercalated discs were not seen in the TG-β1L262R myocardium, in which loss of CPβ1 function caused prominent myofibril dysgenesis. This result does not support the hypothesis that myofibril dysgenesis alone can cause multiple truncated intercalated discs, as seen in TG-wtβ2 myocardium. Alternatively, the multiplicity of intercalated discs may, in TG-wtβ2 mice, be due the increased level of CPβ2, which is a component of the intercalated disc, having dominant effects on other components of the intercalated disc.

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