Supplementary Information for Opitz et al.

DEVELOPMENTALLY REGULATED SWITCHING OF TITIN SIZE ALTERS MYOFIBRILLAR STIFFNESS IN THE PERINATAL HEART

Expanded Materials and Methods

Heart tissue

Pregnant rats (at different gestational stages), non-pregnant adult and neonatal rats were killed by cervical dislocation and fetal rats were killed by decapitation in accordance with institutional guidelines. The hearts were immediately frozen in liquid nitrogen and stored at -80^{o}C. Whole hearts (prenatal rats) or left ventricles (postnatal rats) were used for further analysis. Elsewhere we reported that freezing does not alter the passive mechanical properties of the samples.[1]

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis and western blotting

2% SDS-PAGE and immunoblotting, optimized for detecting proteins in the megadalton range, were performed as described[2,3] using a Laemmli buffer system.[4] Protein bands were visualised with Coomassie brilliant blue R. Adult rabbit soleus muscle was loaded on each gel to provide known molecular-weight bands for titin (3700 kDa) and nebulin (~800 kDa). Calibration of gel-band intensities and molecular weights was done by generating calibration curves as described in Ref.3. Gels were digitized and densitometric analysis was performed using TotalLab software (Phoretix, Newcastle upon Tyne, UK). Immunoblotting was done with a chemiluminescent reaction kit (ECL system, Amersham Pharmacia) according to standard protocols.[3]

Antibodies

The following titin antibodies were used: MG1 to N2BA-titin[5], and I25[6], I27[7], N2B[6], I84/86[8] and BD6[9] to all cardiac titin isoforms. Anti-obscurin antibody I48/I49[10] and a 1:1 mixture of the antibodies anti-nebulin serine-rich domain and anti-nebulin M176-M181[11] were used for western blotting. We thank Drs. M. Gautel and S. Labeit

for generously providing antibodies. Cy3-conjugated IgG (for IF) and peroxidase-conjugated IgG (for western blotting) served as secondary antibodies.

RT-PCR

Total RNA was isolated from rat-heart tissue at stages e10, e16, 1d, 7d, 16d, and adult using Trizol (Invitrogen) according to standard protocols. cDNA was synthesized from 1µg total RNA with the Superscript™ Choice System for cDNA synthesis (Invitrogen) using random hexamers. RT-PCR primers were designed with a computer program (Primer Express, 2.0). Primer sequences are shown in Table 1. *Rattus norvegicus* sequences[12] were compared with the human genomic sequence of titin (Acc. No. AJ277892),[13] in order to find exon boundaries and the lengths of intermitting introns. Primers were designed across exon boundaries to ensure that no genomic DNA was amplified.

Table 1. Primer pairs used (cf., Fig. 2A and Greaser et al., 2002).[13]

Exons	Sense primer (5'-3')	Antisense primer (5'-3')	GeneBank Acc. No.
Exon 49-50	CCAAGCTCACTGTGGGAGAAA	GCTACTTCCAAGGGCTCAATTC	AF525412
Exon 50-219	CCAACGAGTATGGCAGTGTCA	TGGGTTCAGGCAGTAATTTGC	AF525412
Exon 108	CAAAGCGCATACAGAACATCGT	GTCAAAGGACACTTCACACTCAAAA	AF525411
Exon 107-108	CGGCAGAGCTCAGAATCGA	GTCAAAGGACACTTCACACTCAAAA	AF525411
Exon 50-71	CCAACGAGTATGGCAGTGTCA	ACTACAGGCGGAAAGCTACTAAAAAC	AF525411
Exon 50-80	CCAACGAGTATGGCAGTGTCA	GGAGAGCCACTTATCTTGCATTC	AF525411
Exon 50-91	CCAACGAGTATGGCAGTGTCA	CCCTGGAGCTCGCACTCA	AF525411

Quantitative real-time RT-PCR was performed using 25µl SYBR Green PCR Mastermix (Applied Biosystems), 3µl 5µM forward and reverse primer each, 1µl cDNA template and 18µl H_20. Real-time RT-PCR was conducted in an ABI 7000 thermal cycler. The protocol consisted of 2 min at 50°C, 10 min at 95°C and then 40 cycles of incubation at 95°C for 15 seconds followed by one minute at 60°C. The RT-

PCR primers used all worked with 90-110% efficiency, which allows the reactions to be compared to one another (Invitrogen, real-time qPCR and qRT-PCR protocols). Data acquisition and analysis were performed with ABI Prism 7000 SDS software. Both melting-curve and agarose-gel analysis (Fig.3A in main text) confirmed that the primer pairs used amplified only one product. Whereas standard curves are widely used for calculating amplification, a recent evaluation of this method[14] showed that it is less erroneous and more practical to directly compare experimental samples against controls. In addition it was reported[15] that directly comparing one splice variant to another alleviates the need for an internal control because the control will be cancelled from the ratio between the two splice variants. Therefore, the different titin-mRNA splice variants were directly compared to one another in the present study.

Sample preparation for mechanical measurements

Myofibrils or muscle-cell fragments of adult rat myocardium to be used for force measurements or IF were prepared as described.[16,17] Briefly, thin muscle strips were dissected, tied to thin glass rods and skinned in icecold "rigor" solution (no ATP present) in the presence of 0.5% Triton X-100 for ≥4 hours. To obtain myocyte fragments, the strips were minced and homogenized in rigor solution. Fetal and neonatal heart tissue was homogenized in relaxing buffer (ATP present) by passing through a syringe. All solutions were supplemented with 40 μg/ml protease inhibitor leupeptin to minimize titin degradation.

Immunofluorescence microscopy

IF measurements on stretched myofibrils were performed with the aid of hydraulic micromanipulators (Narishige, Japan) under a Zeiss Axiovert 135 inverted microscope (epifluorescence mode; 100x oil immersion, 1.4NA objective) as described.[6,18] Myofibril images were recorded with an integrating color-CCD camera (Sony), frame grabber, and Scion Image software (NIH, Bethesda, MD).[6] Myofibrils were stretched in relaxing solution containing active-force inhibitor BDM (20mM) and protease inhibitor leupeptin (40μg/ml) at room temperature from slack SL to a desired length, labeled with a primary antibody and, after washout, with Cy3-

conjugated anti-mouse or anti-rabbit IgG. Myofibrils stained with secondary antibody only showed no fluorescence. The analysis of titin antibody-epitope spacing was done as described previously.[6] To detect the Z-lines in each myofibril, IF images were compared with the corresponding phase images.

Force measurements

Skinned cell fragments (diameter, 5-8μm) were suspended between micromanipulator-positioned glass needles attached to a piezoelectric micromotor (Physik Instrumente, Karlsruhe, Germany) and a fibre optic-based force transducer (homebuilt) with nanonewton resolution.[1,2] Data collection and motor control were done with a PC, data acquisition board, and custom-written LabView software (National Instruments, Austin, TX). Force data were related to cross-sectional area inferred from the diameter of the specimens.[19] Passive force of nonactivated myocytes was measured at room temperature in relaxing buffer containing 20mM BDM and 40μg/ml leupeptin. Force was recorded (sampling rate, 2 kHz) in protocols in which specimens were extended step-wise from slack SL. The stretch amplitude per step was 50-300nm/sarcomere; a given stretch step was completed in 10-60ms. Following each step, the specimen was held at a constant SL for at least 4s to wait for stress relaxation. To test for possible shifts of force baseline, myofibrils were released back to slack SL.

Modeling titin-based stiffness and force-dependence of epitope positions based on SDS-PAGE results and mechanical parameters of titin-domain function

The force-extension curve for a given titin isoform in the extensible I-band region of the half-sarcomere was modeled as three wormlike chains (WLCs)[20] acting independently and in series. Evidence suggests that these correspond to segments of tandem-Ig, the PEVK region and the N2B-unique sequence (uN2B).[21] Each was characterized by different persistence and contour lengths such that the total titin extension was given by:

$$X = \sum_{i=1}^{3} x_i \quad .$$

The extension x_i at force F of the i-th spring satisfies (WLC model):

$$F = \frac{k_B T}{L_{pi}} \left(\frac{1}{4(1 - x_i / L_{ci})^2} + \frac{x_i}{L_{ci}} - \frac{1}{4} \right) \quad .$$

The Boltzmann constant is denoted as k_B, the absolute temperature as T, with L_{ci} and L_{pi} the contour and persistence lengths, respectively, of the i-th spring. Solutions for the non-linear equations were found using numerical interpolation. Applying the same approach as Li et al. (2002),[21] unfolding of Ig domains (a rare event *in situ* under normal conditions; Ref. 22) was modeled as either a 2-state process (folded and unfolded) for I1-I15 (the "proximal" domains), with a probability of $P_{prox}=A/(A+B)$ where A and B are force-dependent unfolding and folding rate constants, or as a 3-state process (folded, intermediate and unfolded) for I84-I105 (the "distal" domains), with a probability of $P_{dist}=A_1A_2/(A_1A_2+B_1B_2+A_1B_2)$, with A_1 and B_1 being force-dependent unfolding and folding rate constants between the fully-folded state and an intermediate state, and A_2 and B_2 the equivalent rate constants between the intermediate state and the fully-unfolded state. For N2BA-titin isoforms, the kinetic properties of any Ig domains in the differentially spliced region of I28-I79 (the "mid-Ig" domains; Ref. 13) and in the N2-B/N2-A segments were assumed to be identical to those of the distal Ig domains. The values of parameters used are shown in Table 2. If an Ig domain unfolds, the contour length of the tandem-Ig wormlike chain is assumed to decrease by 4.4 nm, the length of a single folded Ig,[23] with a simultaneous increase in the contour length of the wormlike chain of 32.5 nm.

High resolution SDS-PAGE and western blotting suggested titin in adult rat hearts was almost exclusively the N2B-isoform whereas that in fetal rat hearts (e16) was exclusively a very long N2BA-1-isoform, with molecular weights of 3.0MDa and 3.7MDa, respectively. The difference in molecular weight between the two was assumed to be due to extra insertions of either PEVK or mid-Ig domains in the N2BA-isoform.[12,13] This allowed an estimate for the total Ig-domain insertion given 10-12 kDa for each extra Ig domain, assuming a certain total PEVK length.

Table 2. Parameters used in the prediction of the force-extension relations and antibody-epitope mobility.

N2B-titin isoform (M_w=3.0 MDa)

	A/ s^{-1}	B/ s^{-1}	A_1/ s^{-1}	B_1/ s^{-1}	A_2/ s^{-1}	B_2/ s^{-1}	n	L_p/ nm	L_c/ nm
proximal Ig's	-	-	1.0×10^{-2}	100	3.3×10^{-3}	0.33	15	10	66
distal Ig's	8.0×10^{-5}	1.2	-	-	-	-	22	10	97
mid Ig's (N2-B)	8.0×10^{-5}	1.2	-	-	-	-	4	10	18
PEVK residues	-	-	-	-	-	-	184	0.91	66
uN2B residues	-	-	-	-	-	-	526	0.66	189

N2BA-1-titin isoform (M_w=3.7 MDa)

*	A/ s^{-1}	B/ s^{-1}	A_1/ s^{-1}	B_1/ s^{-1}	A_2/ s^{-1}	B_2/ s^{-1}	N	L_p/ nm	L_c/ nm
proximal Ig's	-	-	1.0×10^{-2}	100	3.3×10^{-3}	0.33	15	10	66
distal Ig's	8.0×10^{-5}	1.2	-	-	-	-	22	10	97
mid Ig's	8.0×10^{-5}	1.2	-	-	-	-	48	10	211
PEVK residues	-	-	-	-	-	-	2179	0.91	784
uN2B residues	-	-	-	-	-	-	526	0.66	189

* The number (n) of extra mid-Ig's has been deduced by assuming that the extra molecular weight after inclusion of additional PEVK of 1995 a.a. for the N2BA-1-titin isoform is due solely to additional Ig domains. Contour lengths for the PEVK and uN2B regions have been calculated on the basis of 0.36nm per residue, with the length for the Ig regions being 4.4nm per domain. As the persistence lengths for distal and proximal Ig segments are considered equal, they can be represented as a single wormlike chain whose contour length is the sum of those for the two separate regions. For the model, mid-Ig's were assumed to have properties equivalent to distal Ig's. All kinetic parameters are taken from data published in Li et al. (2002).[21] Titin sequence information is from Freiburg et al. (2000)[13] and Greaser et al. (2002).[12]
For the N2BA-2-titin isoform (M_w~3.5 MDa), we assumed an additional 30 mid-Ig domains and 1216 PEVK residues, compared to the N2B-isoform.

Passive-tension (T_p) data obtained from skinned myocyte fragments of adult and fetal (e16) rat hearts were modeled. The predicted force-extension relation for a single molecule of each titin isoform was multiplied by a fixed scaling-factor, N, of 2.4×10^9 titin molecules/mm^2, obtained from EM data,[24] and a variable area-factor, α, to account for the proportion of cross-sectional area of the myocyte occupied with myofibrils (a large proportion of cardiac-myocyte volume is non-myofibrillar elements). One sarcomere-length was modeled to consist of two extensible I-band regions of titin and inextensible Z-disk (~200nm) and A-band (~1.6μm) regions. A small variable extension offset X_{offset} was applied to the fit to account for imprecision in SL measurements. The fitting algorithm was written in Matlab using Nelder-Mead Simplex minimization:[25]

$$T_P = \alpha NF\left(X - X_{offset}\right).$$

T_p data from adult rat-heart cells were best fitted using an N2B-titin isoform in the prediction algorithm with an extension offset of -10nm and α=0.50. Best fits for fetal heart (e16) myocyte data were obtained using an N2BA-1-titin isoform with a total PEVK content roughly equivalent to that of the 3.7MDa soleus-muscle titin isoform (an additional 1995 residues compared to the N2B-isoform) and an extra 44 mid-Ig domains, with an extension offset of +20nm and α=0.39. The values of α were consistent with previous estimates for myofibrillar volume fraction estimated from cross-sections of fiber bundles taken from fetal heart,[26] which suggested that no more than ~40% of the total cross-sectional area of a cardiomyocyte is myofibrils, compared with ~50% in adult heart.[27]

Epitope positions for the anti-titin antibodies I27, MG1 and I84/86 were predicted as a function of SL using the same force-extension model, and compared against IF data. The model was used as in Li et al. (2002)[21] to predict the separate extensions of the proximal, mid-Ig, distal, PEVK and uN2B regions as a function of force, and then numerical interpolation was used to generate a functional relationship to SL. The distance of the I27 epitope from the center of the Z-disk was assumed to be

the sum of the extensions of the proximal and uN2B regions plus half the (inextensible) Z-disk width (~100nm). Similarly, the Z-disk-center to MG1-epitope distance was taken as the sum of the extensions of the proximal, uN2B and mid-Ig regions plus half the Z-disk width. The I84/86-epitope distance to the Z-disk center was assumed to be the sum of the extensions of the proximal, uN2B, mid-Ig and PEVK regions plus half the Z-disk width.

Finally, to obtain the extensions of the differentially spliced titin-segments (mid-Ig, PEVK) in the individual isoforms (N2BA-1, N2BA-2, N2B), the modeled antibody epitope-mobility curves were subtracted from one another as follows:
mid-Ig extension: MG1-curve minus I27-curve (e16, 1d myofibrils)
PEVK extension: I84/86-curve minus MG1-curve (e16, 1d myofibrils)
I84/86-curve minus I27-curve (1d, adult myofibrils) = N2B-PEVK.

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